Takuji Sasaki · Motoyuki Ashikari
Editors

Rice Genomics, Genetics and Breeding

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Preface

Rice is a staple food for about half of the world population and has sustained mankind since the dawn of agriculture. As the mid-twenty first century approaches, the world is faced with the monumental task of feeding 9 billion people. To address this challenge, sustainable increases in cereal grain production has to be met. Increasing rice production would require significant improvements in two key aspects: (1) crop management strategies including soil, water, and pest and disease management and (2) development of cultivars with better characteristics including higher yield and improved grain quality. Both these factors would be critical in realizing sustainable rice cultivation.

This book focuses on the body of genetics, genomics, and breeding researches that have been carried out to characterize the rice plant itself towards the purposeful development of better rice cultivars. Existing rice varieties that are being grown in localities worldwide are results of breeding efforts in response to demands by farmers for cultivars that are adapted to specific regional environments and by consumers with established quality preferences. For a long time, breeding for improved cultivars relied on visual observations and selection of recombinants with favorable phenotypes. With the discovery of Mendel's law, however, it has been shown that many important phenotypes or traits are too complex to be explained by mere visual observations.

As in the case of many other organisms, advancement in molecular genetics has revolutionized our understanding of rice as a plant. The map-based, genome sequence of a standard rice variety that was released in 2004 via an international collaboration has facilitated the identification and cloning of genes and quantitative trait locus (QTL) controlling various traits. This sequence information also opened the doors to the field of genomics which aims to understand genome-wide variation in rice in terms of gene expression, metabolite profile, and hormonal level during development. These information are in turn used to elucidate genetic networks that make up the rice plant.

Combined with the fast pace of technological advancements, the mega-volume of genomic information has allowed targeted genetic manipulations to induce variations in a given allele using genome-editing technologies such as TALENS or CRISPR/Cas9 system. To this day, genome editing has found applications not only in validating gene function but also in generating new alleles that can give more favorable phenotypes.

This book is composed of 28 chapters that describe the recent progress and future perspectives in *Rice Genomics, Genetics and Breeding*. Each chapter, written by established rice researchers who are experts in their field, is a comprehensive look at the genetics and genomics machineries underlying various traits in rice that can be used to address issues on food security. This is especially intended for rice scientists, breeders, post-docs, and graduate and undergraduate students as a standard reference that can be used to device strategies towards solving the 9 billion people challenge.

The editors would like to gratefully acknowledge the respective authors for their outstanding contributions towards the realization of this wonderful book.

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Chapter 1
Genome Sequences of *Oryza* Species

Masahiko Kumagai, Tsuyoshi Tanaka, Hajime Ohyanagi, Yue-Ie C. Hsing, and Takeshi Itoh

Abstract This chapter summarizes recent data obtained from genome sequencing, annotation projects, and studies on the genome diversity of *Oryza sativa* and related *Oryza* species. *O. sativa*, commonly known as Asian rice, is the first monocot species whose complete genome sequence was deciphered based on physical mapping by an international collaborative effort. This genome, along with its accurate and comprehensive annotation, has become an indispensable foundation for crop genomics and breeding. With the development of innovative sequencing technologies, genomic studies of *O. sativa* have dramatically increased; in particular, a large number of cultivars and wild accessions have been sequenced and compared with the reference rice genome. Since de novo genome sequencing has become cost-effective, the genome of African cultivated rice, *O. glaberrima*, has also been determined. Comparative genomic studies have highlighted the independent domestication processes of different rice species, but it also turned out that Asian and African rice share a common gene set that has experienced similar artificial selection. An international project aimed at constructing reference genomes and examining the genome diversity of wild *Oryza* species is currently underway, and the genomes of some species are publicly available. This project provides a platform for investigations such as the evolution, development, polyploidization, and improvement of crops. Studies on the genomic diversity of *Oryza* species, including wild species, should provide new insights to solve the problem of growing food demands in the face of rapid climatic changes.

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1.1 Introductory Overview of Rice Genome Sequencing

The central dogma of molecular biology suggests that all the biological processes of an organism should derive from the information encoded in its genomic DNA. As the genome is considered a blueprint of cellular life forms, knowledge of the entire genome sequence should be equivalent to understanding the whole biological mechanism. In the 1980s, biologists envisaged that genome-wide sequencing would expedite molecular biological studies to a much greater extent than the piecemeal analyses of a handful of genes (Dulbecco 1986). However, the sequencing technologies available at that time were not sufficient to determine billions of nucleotides in a short time. Therefore, to achieve rapid whole-genome sequencing of higher eukaryotes, several innovative sequencing technologies have been developed in the twenty-first century. In this chapter, we provide an overview of the history of genome sequencing in *Oryza* species over the last two decades, which was strongly affected by the advent of novel sequencing platforms.

Since the completion of the genome sequencing of *Haemophilus influenzae* in 1995 (Fleischmann et al. 1995), genome-wide sequencing has played a pivotal role in current biology, although the era of genomics emerged slightly later for plants than microbes and animals. Sequencing using the Sanger method was neither massive nor fast for large genomes, and the determination of higher eukaryote genomes therefore generally took a long time. Although the genome sequence of *Arabidopsis thaliana*, the best studied model plant, was published in 2000 (Arabidopsis Genome Initiative 2000), genome-wide sequence data for crops were not available until 2002, when the genomes of *japonica* and *indica* cultivars of rice (*O. sativa* L.) were deciphered using the whole-genome shotgun method (Goff et al. 2002, Yu et al. 2002). However, these two genome sequences were extensively fragmented, reflecting the limitations of the sequencing strategy, and a much more accurate genome sequence that would meet the demands for further genomic studies was therefore anticipated. Long before the publication of the genomes obtained using the shotgun method, other efforts toward rice genome sequencing were conceived and initiated in the early 1990s in Japan (Sasaki 1998); however, these techniques were not sufficient to sequence the complete genome at that time. In 1998, the International Rice Genome Sequencing Project (IRGSP) was organized by pioneering researchers from ten countries and regions, including Japan (Matsumoto et al. 2016). This international collaborative project employed precise genome sequencing based on a physical map of P1-derived artificial chromosome (PAC)/bacterial artificial chromosome (BAC) clones to generate a high-quality genome sequence. In fact, the resultant genome sequence published in

2004 (International Rice Genome Sequencing Project 2005), which was later improved in 2013 (Kawahara et al. 2013), is accurate and still serves as the essential foundation of cereal genomics (Matsumoto et al. 2016).

Since the public release of the IRGSP genome, next-generation sequencing (NGS) technologies have dramatically altered molecular biological studies. Genome sequencing is now rather easier and more cost-effective than gene-by-gene approaches. For example, the causative mutation of a phenotype of interest may be more rapidly observed by sequencing than through other conventional molecular genetic methods. Such high-throughput sequencing studies are particularly effective in rice because we can map newly sequenced reads to the high-quality reference genome. In addition, while current sequencing technologies are suitable for massive sequence production, these data generally contain a significant number of errors, several orders of magnitude larger than the errors generated using the Sanger method. NGS-based genome sequencing is currently widely used for resequencing, as observed in rice, for which we have an appropriate reference genome for comparison.

To construct a high-quality reference genome, yet another innovative technology is needed. The single-molecule real-time sequencing technology of Pacific Biosciences (PacBio) (Eid et al. 2009) is currently a promising method for de novo genome sequencing and is therefore being applied to crop species (Sakai et al. 2015; Du et al. 2017), but the assemblies presented thus far still contain some gaps. Another emerging platform is the MinION device, a single-molecule nanopore sequencer from Oxford Nanopore Technologies (Michael et al. 2017). MinION is highly cost-effective and presents a strong capability for sequencing in the field, leading to its widespread usage. In addition to these methods, the combination of Illumina with Hi-C, which can reutilize sequence data generated in the past, exhibits great potential (Dudchenko et al. 2017). Therefore, since low-cost/high-quality de novo genome sequencing is anticipated in the near future, multiple reference genomes of *japonica* cultivars, *indica* cultivars, and wild rice accessions will be available, and rice genomic research will be further accelerated (Fig. 1.1).

1.2 Rice Genome Sequencing Projects and the Release of the IRGSP Reference Genome

Two rice draft genomes, one for *japonica* and the other for *indica*, were made available prior to the completion of IRGSP. Beijing Genomics Institute (BGI) sequenced an *indica* cultivar, representing the major cultivar group in China and the southern part of Asia (Yu et al. 2002). They sequenced the genome of cultivar 93-11, the parental cultivar of super-hybrid rice Liang-You-Pei-Jiu (LYP9). Two other groups, Monsanto Co. and Syngenta Co., sequenced the *japonica* cultivar Nipponbare, which was the same cultivar sequenced by IRGSP (Barry 2001; Goff et al. 2002). These draft genome sequencing projects employed whole-genome shotgun sequencing,

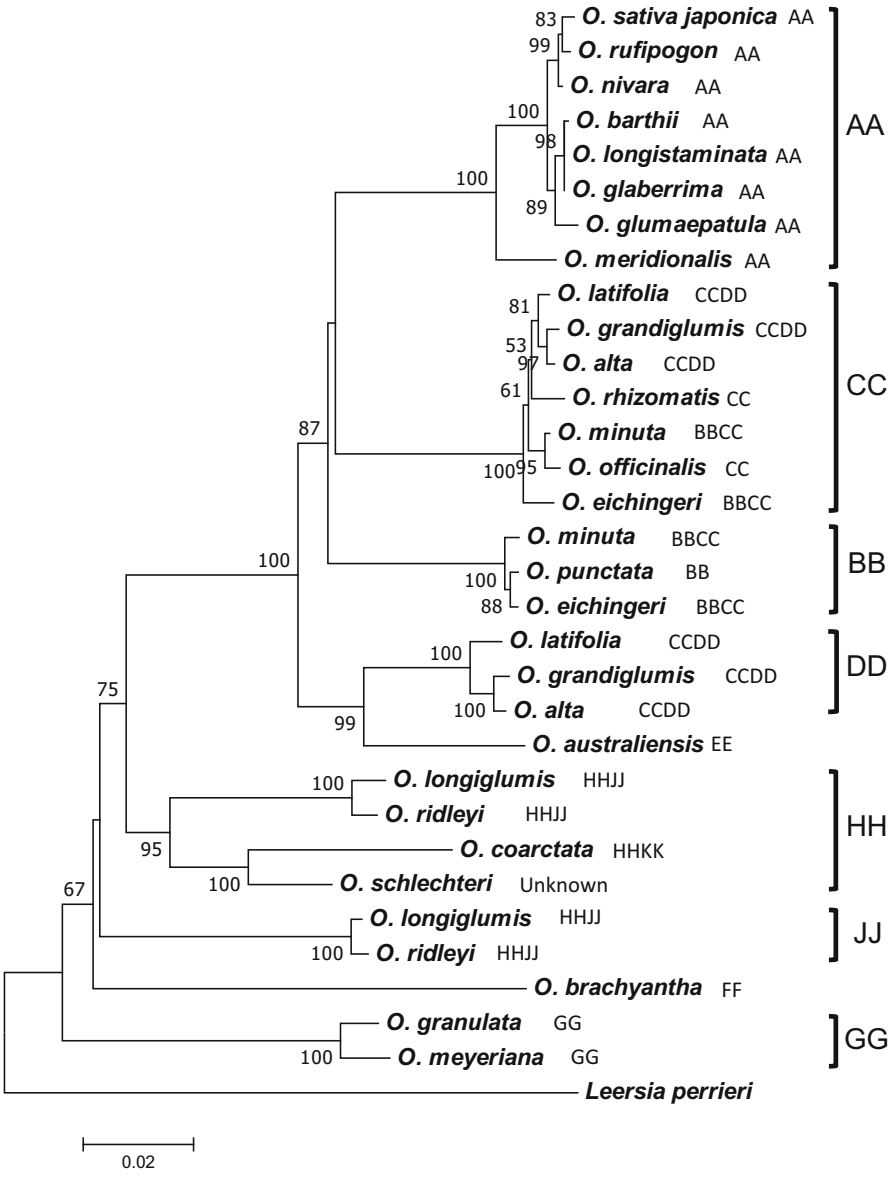

Fig. 1.1 Phylogeny of the genus *Oryza* based on the nucleotide sequences of *Adh1* (Ge et al. 1999). The tree was inferred using the neighbor-joining method, and the evolutionary distances were computed using Kimura's two-parameter method. The bootstrap test values (1000 replications) are shown next to the branches. The genome type of each accession is indicated with two or four letters. The estimated ancestral diploid genome of each allele was inferred from the tree topology and is indicated on the right side of the tree (Ge et al. 1999). This analysis was conducted using MEGA7 (Kumar et al. 2016)

in which Sanger sequencing reads were assembled without anchoring to chromosomes. Therefore, a draft genome could be rapidly constructed. However, the quality was not comparable to other reference genomes, such as those of *Arabidopsis*, yeast, *Drosophila*, and *C. elegans*. The assembled genome sequences were divided into 791 BAC contigs in *japonica* and 103,044 scaffolds in *indica*. While comparative genomics and gene analysis could be performed using these genome data, a high-quality rice reference genome sequence was desired by the rice research community as well as researchers focused on cereal crops and other plants.

IRGSP published the whole-genome sequence of a *japonica* cultivar in 2004 (IRGSP 2005). The genome was sequenced using map-based sequencing and clone-by-clone Sanger sequencing. The BACs, PACs, and fosmid clones in the physical map were sequentially sequenced, independently assembled, and used for reconstruction of the genome assembly. Genomic libraries from Nipponbare, a temperate *japonica* cultivar that is widely used for experiments, were employed to establish the physical map. Sequence gaps were cautiously resolved by selecting gap-bridge clones and PCR fragments and through the direct sequencing of BACs. Each clone was sequenced via shotgun sequencing with tenfold coverage. The quality of the obtained assembly was expected to exceed the 99.99% accuracy standard (less than one error in 10,000 bases). The finished genome provided direct evidence of a rice genome size of 389 Mb, which is three times larger than the *Arabidopsis thaliana* genome. The completed IRGSP genome sequence was 370 Mb in length, representing 95% coverage of the rice genome. However, comparative analysis against the two previously published draft genome sequences showed that the coverage of these draft genomes compared with the IRGSP genome was 69% and 78% in *indica* and *japonica*, respectively. Ab initio gene finding predicted a total of 37,544 non-transposable-element-related protein-coding sequences, and 2859 rice genes were not previously observed in the *Arabidopsis* genome. The completeness of the IRGSP genome enabled an analysis of centromeres in the untraversed genomic region. Deciphering these centromere sequences was a remarkable effort. The entire centromere sequences of chromosomes 4 and 8 were determined, showing sizes of 59 and 69 kb, respectively, based on clustered CentO repeats (Nagaki et al. 2004; Wu et al. 2004; Zhang et al. 2004). *Oryza sativa* became the first eukaryotic species with a complex genome structure whose complete centromere sequence was analyzed. The high-quality map-based genome sequence of Nipponbare remains the only monocot genome and serves as the role model for genome sequencing projects in other cereal crops with large genomes and complex chromosome contents (Matsumoto et al. 2016).

1.3 Genome Annotations and the Release of the Revised Genome Assembly, IRGSP-1.0

Genome annotation is absolutely essential for utilizing genome information in biological studies. Prior to completing the IRGSP genome sequencing project, The Institute for Genomic Research (TIGR), a member of the IRGSP, initiated a gene annotation project, currently known as the Rice Genome Annotation Project (RGAP), and has successively released the results to the research community (Yuan et al. 2003). This annotation database is now maintained by Michigan State University (MSU, http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). IRGSP members launched the official genome annotation project, the Rice Annotation Project (RAP), an initiative of the National Institute of Agrobiological Sciences (NIAS) in Japan, and the data are released from RAP-DB (http://rapdb.dna.affrc.go.jp, Ohyanagi et al. 2006). RAP-DB is currently maintained by National Agriculture and Food Research Organization. RAP adopted evidence-based annotation; therefore, representative genes are associated with experimental results, such as full-length rice cDNA sequences, rice ESTs (expressed sequence tags), and mRNA/protein sequences from closely related species. In the case of ESTs, several ab initio gene prediction programs are combined to generate intact gene structures. In addition, RAP has continued improving the reference genome assembly and is currently manually curating gene structures and functional descriptions based on the literature. In 2013, the most recent genome assembly, Os-Nipponbare-Reference-IRGSP-1.0 (IRGSP-1.0), was published as a joint effort of the RAP and RGAP annotation projects (Kawahara et al. 2013). This unified genome represents the revised version of these projects, based on the use of NGS technology and the minimal tiling path of clones with optical mapping. Resequencing data from two Nipponbare individuals obtained from the Illumina Genome Analyzer II/IIx platform and Roche 454 GS FLX was used for correcting single nucleotide and short insertion/deletion errors (Kawahara et al. 2013). After mapping the NGS reads to the reference genome, a total of 4886 single-nucleotide sequencing errors and five insertions/deletions were detected in the whole Nipponbare genome. This result validated the quality of the original assembly, showing an average error rate of 0.15 per 10,000 nucleotides (Kawahara et al. 2013). The size of revised Nipponbare genome was estimated to be 384.2–386.5 Mbp based on revised assembly and gap size estimation by fluorescence in situ hybridization and estimated length of the rDNA regions. After the release of IRGSP-1.0, the two annotations, RAP and RGAP, can be compared directly on the new assembly.

1.4 De Novo Assembly of the *indica* Genome via NGS

Because the map-based genome sequence of IRGSP was obtained using the *japonica* cultivar and because the draft genome of *indica* was far from completion, determining the genome sequence of *indica* remained one of the major goals. According to studies on genetic diversity and molecular phylogeny, significant genetic diversity of *O. sativa* species has been observed. For example, *japonica* and *indica* originated from different ancestral populations of the wild rice species *O. rufipogon* (Cheng et al. 2003; Londo et al. 2006). Therefore, there are many structural differences between *japonica* and *indica*, including structural variations at the chromosomal segment level. Moreover, high within-population genetic diversity has been observed in *indica*, and at least two varietal groups (*indica* and *aus*) were defined based on classical observation of physical and physiological traits, which were subsequently confirmed based on modern genetics and genomic data (Glaszmann 1987; Garris et al. 2005; McNally et al. 2009). Hence, researchers demanded genome sequences for *indica* varietal cultivars. Thus far, several de novo genome assembly studies in *indica* varieties, including the *aus* group, have been performed using NGS technologies (Sakai et al. 2014; Schatz et al. 2014; Du et al. 2017). Although the strategy for these de novo assembly studies primarily involves whole-genome shotgun sequencing, similar to past draft genome sequencing projects, computational algorithms for assembling the genome sequence enabling researchers to construct a higher-quality genome have been developed and applied under some NGS methods, such as those involving mate pair libraries and ultra-long read sequencing (e.g., the PacBio sequencing platform, whose read length N50 (a statistic defined as the shortest sequence length for the top 50% of sequences) can be 10 kb and more). In particular, long sequence reads generated using a third-generation sequencer, such as the PacBio platform, are powerful for de novo genome assembly in species with large complex genomes (Sakai et al. 2015). This technique has frequently been used in recent de novo genome assembly studies and employed for *indica* genome assembly (Du et al. 2017). Regarding the comparative quality of the obtained genomes, the first-draft genomes generated using Sanger sequencing exhibited N50 sizes of 6.69 kb for contigs (which is the minimum unit of genome assembly) and 11.76 kb for scaffolds (Yu et al. 2002), while the most recently reported *indica* de novo assembly using PacBio reads showed N50 sizes ranging from several hundreds of kb to 1.1 Mb (which varies based on the assembly software used) for contigs and 2.48 Mb scaffolds (Du et al. 2017).

1.5 *O. sativa* Genome Resequencing Project

Reflecting the dramatic reduction in sequencing costs after the emergence of NGS technologies, many genome sequencing studies of cultivated rice have been performed in this decade. With the improvement of sequencing platforms and chemistry, accompanied by reduced costs, we have obtained an increasing amount of individual whole-genome data. The scale of genome sequencing studies has gradually expanded. Reference mapping-based studies of *O. sativa* for detecting single-nucleotide variants (SNVs) of each variety were initiated on a small scale, involving one to a few samples, which then increased to dozens and hundreds of samples, finally reaching thousands of genomes (Yamamoto et al. 2010; Huang et al. 2010; Xu et al. 2012, The 3000 rice genomes project 2014, Yano et al. 2016). These investigations were primarily aimed at understanding the relationship between genotype and phenotype based on genome-wide association studies (GWAS) and provided information such as variety-specific genetic polymorphisms, within- and between-population genetic diversity, and insights into the history of Asian rice domestication. The population genomic analyses of several samples enabled the detection of footprints of artificial selection in past domestication and breeding efforts. A large-scale genome resequencing study involving more than 1000 accessions of *O. sativa* in China and its wild ancestor *O. rufipogon* revealed that many causal genes for domestication-related phenotypes, such as grain shattering, grain size, plant architecture, and grain color, were located in candidate regions for selective sweeps (Huang et al. 2012). This successful work indicated the usefulness of determining whole-genome polymorphisms for large-scale sample collection and the detection of selective sweeps using population genetic statistics to identify candidate gene alleles related to beneficial traits. The most recent large-scale sequencing project for *O. sativa* is the 3000 Rice Genomes Project. This ongoing project has resequenced a core collection of 3000 rice germplasm accessions, including both *japonica* and *indica* cultivars, selected from resources of the International Rice Research Institute (IRRI) and the Chinese Academy of Agricultural Sciences (CAAS), comprising accessions from 89 countries distributed in Southeast Asia (33.9%), South Asia (25.6%), and China (17.6%). Each genome of 3000 accessions contained sequences with 14X genome coverage on average, indicating that this amount of data provided an adequate depth for the detection of reliable SNVs, with 17TB of data being obtained using the Illumina platform in total. Based on reference mapping to IRGSP-1.0, approximately 18.9 M single-nucleotide polymorphisms (SNPs) were identified (The 3000 rice genomes project 2014). These data will serve as a fundamental resource for the discovery of novel alleles for important phenotypes that are useful for rice improvement and adaptation to changing environments.

1.6 Domestication History of *O. sativa* and Contribution of Genomic Studies

The domestication history of crop species has attracted much attention from a variety of research fields. Understanding the domestication process is a subject of much interest and will contribute to the management of next-generation agriculture in the coming era, with ongoing, rapid environmental change. Recent efforts in genomic studies have shed light on the history of Asian rice domestication. The mystery of the origin and domestication process of Asian cultivated rice has been argued for decades. The main issues were the origins of *japonica* and *indica* and whether these varietal groups were independently domesticated. Previous genetic studies employing *O. sativa* and a diverse panel of *O. rufipogon* indicated that *japonica* and *indica* showed close affinity to different *O. rufipogon* populations and suggested multiple origins of *O. sativa*, with *japonica* originating in China and *indica* originating in South/Southeast Asia (Cheng et al. 2003; Londo et al. 2006; Rakshit et al. 2007), and their divergence time predated the onset of domestication (Ma and Bennetzen 2004; Vitte et al. 2004; Zhu and Ge 2005). This hypothesis presented a good fit for archaeological data demonstrating the existence of old rice culture ruins in the Yangtze River basin in China and the Ganges River basin in India (Fuller 2006). However, the story is more complicated, as some population genetic studies employing a larger number of loci have suggested nonindependent domestication of *japonica* and *indica* (Gao and Innan 2008; Molina et al. 2011). Recent studies based on whole-genome data clarified the detailed process of Asian rice domestication, showing that *japonica* and *indica* exhibit divergent genomic backgrounds, coming from different wild rice populations, and that gene introgression of domestication-related genes from *japonica* to *indica* has occurred (Huang et al. 2012; Yang et al. 2012). Furthermore, two *indica* rice varieties (*indica* and *aus*) also have different origins (Civáň et al. 2015; Choi et al. 2017). Phylogeographical analysis of worldwide wild rice panels comprising more than 400 accessions, together with cultivated rice, demonstrated that wild rice collected from the middle of the Pearl River region in southern China showed the closest genetic affinity to cultivated rice in domestication-related gene regions (Huang et al. 2012).

1.7 Sequencing of *O. glaberrima*, African Cultivated Rice

In addition to Asian rice, African cultivated rice, *O. glaberrima*, was independently domesticated from the wild rice species *Oryza barthii* in West Africa approximately 3000 years ago (Linares 2002). *O. glaberrima* is well adapted to cultivation conditions in Africa but presents a lower yield potential than that of *O. sativa* (Linares 2002). An early *O. glaberrima* genome sequencing effort resulted in a partial genome-wide sequence of this species (Sakai et al. 2011). To more precisely

determine the genome, a BAC library was prepared to construct a minimum tilling path (MTP) (Wang et al. 2014). The MTP hybrid BAC pools were then subjected to sequencing using Roche/454 technology, with a sequence coverage of 31X. The reference sequences for 12 chromosomes, with the total assembly size of 316 Mb, indicated that *O. glaberrima* was domesticated in a single region along the Niger River (Wang et al. 2014). Comparative genomic analysis highlighted the independent selection of a common set of genes during two geographically distinct domestication processes (Wang et al. 2014). Regarding non-shattering, for example, early domestication-related genes such as *qsh1* (Konishi et al. 2006) and *sh4* (Li et al. 2006) exhibited totally different haplotypes in *O. sativa* and *O. glaberrima* (Wang et al. 2014). Additionally, the *heading date 1* (*Hd1*) gene, related to photoperiod sensitivity, was deleted from *O. glaberrima* (Sanyal et al. 2010; Wang et al. 2014), while single-nucleotide polymorphisms (SNPs) or small insertions/deletions (indels) were responsible for the loss of function of *Hd1* in *O. sativa* (Yano et al. 2000; Takahashi et al. 2009). The accession sequences as well as the assembly size and percentages of repetitive sequences in these African cultivated and wild rice species are listed in Table 1.1.

1.8 Wild *Oryza* Species

The genetic resource center of IRRI maintains a collection of more than 4000 accessions of wild *Oryza* species (Sanchez et al. 2013) comprising 22 relatives found in a wide range of habitats, including areas of Asia, Australia, Africa, and South and Central America, representing 15 million years of evolutionary diversification. This collection includes 14 diploids and 8 polyploids, with genome sizes ranging from approximately 300 Mb to 1.2 Gb. The chromosome number of these accessions is 24 or 48, representing the AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ, and HHKK genome types (Table 1.2, Sanchez et al. 2013). These wild species exhibit significant diversity in terms of morphological traits, plant height, tillering number and stature, flowering behavior, growth habits and panicle, and leaf and seed characteristics. In addition, these species adapt to different habitats and are resistant to a range of biotic and abiotic stresses.

1.9 The International Collaborative for Wild *Oryza* Species Genome Sequencing

An ambitious comparative genomic program entitled the "*Oryza* Map Alignment Project" (OMAP) was established in the early 2000s (Wing et al. 2005). The long-term objective of this work was to generate a genome-level closed experimental system for the genus *Oryza* as a research platform to study evolution, development,

Table 1.1 Current rice reference genomes

Species	Genome type	Accessions	Origin	Assembly size (Mb)	Repeat (%)	References
O. sativa (*japonica*)	AA	Nipponbare	Asia	389	38.9	IRGSP (2005) and Sakai and Itoh (2010)
O. sativa (*indica*)	AA	93-11	Asia	375	42.8	Yu et al. (2002)
O. sativa (*indica*)	AA	Zhenshan 97	Asia	384	41.3	Zhang et al. (2016)
O. sativa (*indica*)	AA	Minghui 63	Asia	386	41.6	Zhang et al. (2016)
O. sativa (*indica*)	AA	Shuhui498	Asia	390	42.1	Du et al. (2017)
O. nivara	AA	W0603	Asia	338	36.4	I-OMAP (2018)
O. rufipogon	AA	W1943	Asia	338	42.1	I-OMAP (2018)
O. glaberrima	AA	IRGC: 96717,	Africa	285	39.3	Wang et al. (2014)
O. barthii	AA	IRGC:105608	Africa	308	38.3	I-OMAP (2018)
O. glumaepatula	AA	GEN1233	America	373	31.4	I-OMAP (2018)
O. meridionalis	AA	W2112	Australia	336	27.2	I-OMAP (2018)
O. punctata	BB	IRGC:105690	Africa	394	49.6	I-OMAP (2018)
O. brachyantha	FF	IRGC:101232	Africa	261	28.7	Chen et al. (2013)
Leersia perrieri	–	IRGC:105164	Africa	267	26.7	I-OMAP (2018)

genome organization, polyploidy, domestication, gene regulatory networks, and crop improvement (Wing et al. 2005). In this project, these researchers constructed and aligned BAC/STS (sequence tagged site)-based physical maps of 11 wild rice species, including 6 diploid genomes (AA, BB, CC, EE, FF, and GG) and 4 tetraploid genomes (BBCC, CCDD, HHJJ, and HHKK) (Wing et al. 2005, Ammiraju et al. 2006, 2010), followed by BAC-end sequencing (Kim et al. 2008). With these resources, the research community revealed gene conversion (Jacquemin et al. 2013), duplication, de novo origination, movement, loss (Zhao et al. 2015), and transposable element (TE) dynamics (Jacquemin et al. 2014). In 2007, this project was transformed into the International *Oryza* Map Alignment Project (I-OMAP) with the aim of generating RefSeq and transcriptome datasets for eight AA genome species and one BB genome species (Jacquemin et al. 2013). These datasets included two Asian AA genome species, *O. rufipogon* and *O. nivara*; one

Table 1.2 Species in the genus *Oryza*

Species	Chromosome number	Genome	Geographical distribution
Oryza sativa complex			
O. sativa	24	AA	All over the world
O. rufipogon	24	AA	Asia, Oceania
O. nivara (also known as annual ecotype of *O. rufipogon*)	24	AA	Asia, Oceania
O. glaberrima	24	AA	West Africa
O. barthii	24	AA	Africa
O. longistaminata	24	AA	Africa
O. meridionalis	24	AA	Australia
O. glumaepatula	24	AA	Central and South America
O. officinalis complex			
O. officinalis	24	CC	Asia
O. minuta	48	BBCC	Philippines
O. rhizomatis	24	CC	Sri Lanka
O. eichingeri	24	CC	Africa, Sri Lanka
O. punctata	24, 48	BB, BBCC	Africa
O. latifolia	48	CCDD	Central and South America
O. alta	48	CCDD	Central and South America
O. grandiglumis	48	CCDD	South America
O. australiensis	24	EE	Australia
O. ridleyi complex			
O. ridleyi	48	HHJJ	Asia, New Guinea
O. longiglumis	48	HHJJ	New Guinea
O. granulata complex			
O. granulata	24	GG	Asia
O. meyeriana	24	GG	Asia
Others			
O. brachyantha	24	FF	Africa
O. schlechteri	48	Unknown	New Guinea
O. coarctata (also known as *Porteresia coarctata*)	48	HHKK	Coastal region of India, Pakistan, and Bangladesh

Australian AA genome species, *O. meridionalis*; one American AA genome species, *O. glumaepatula*; and one African AA genome species, *O. barthii*, the species from which *O. glaberrima* was domesticated. In addition, one African BB genome species, *O. punctata*, and the diploid *Leersia perrieri* as an outgroup species were also added (I-OMAP Consortium 2018). Shotgun sequences with various insert size libraries at a minimum of depth of 100X coverage were generated using Illumina technology for assembly. For the two African species *O. barthii* and *O. punctata*, additional sequence coverage of 10–20X was obtained using Roche/454 technology. Final super-scaffolds were manually constructed using paired BAC-end

sequences and alignment to the Nipponbare sequences as guide information. The total lengths of the resulting genome sequences, with 12 chromosomes in each species, ranged from 267 to 394 Mb (I-OMAP Consortium 2018). The accessions are listed in Table 1.1 with the assembly sizes. Using all 11 species, including the two major Asian cultivars, the African cultivated species, and all wild species mentioned above, several conclusions were reached. Phylogenic analysis indicated that the "crown" age of the AA clade is approximately 2.5 million years, with a rapid diversification rate of ~0.50 net new species/MYR (I-OMAP Consortium 2018). Detailed sequence analysis also showed that extensive introgression has occurred in *Oryza* species, particularly between South American *O. glumaepatula* and the African AA species (I-OMAP Consortium 2018). The turnover rate of LTR retrotransposons within the AA genome lineage was one to two orders of magnitude faster than those estimated for flies and mammals, respectively (I-OMAP Consortium 2018). In addition, thousands of candidate disease resistance genes were discovered in heterogeneous gene pairs organized in a head-to-head fashion, supporting the integrated decoy model for disease resistance (I-OMAP Consortium 2018). The assembled genome sequences and the annotated open reading frame (ORF) amino acid sequences have all been available to the community at Ensembl Plants (http://plants.ensembl.org/index.html) since 2015 and have been extensively employed. For example, using the sequence information for two Asian wild rice species (*O. rufipogon* and *O. nivara*) from I-OMAP, Choi et al. conducted comparative analyses with two *indica* rice varieties (IR64 and 93-11), two *aus* rice varieties (Kasalath and DJ123), and the *japonica* variety Nipponbare (Choi et al. 2017) and concluded that domestication occurred only once, with multiple origins; thus, each domesticated rice subpopulation, including *japonica*, *indica*, and *aus*, arose separately from *O. rufipogon* and/or *O. nivara* progenitors. Furthermore, when Baldrich et al. (2016) analyzed polycistronic miRNAs in the cultivated and wild rice using this resource, they discovered new rice polycistronic miRNAs and suggested that most polycistronic and candidate polycistronic miRNAs showed a pattern of conservation in the genomes of rice species with an AA genome (Baldrich et al. 2016).

1.10 Distantly Related Wild *Oryza* Species

Oryza species that are only distantly related to cultivated rice species in terms of evolution exhibit more diverged morphologies and a wider variety of resistant phenotypes related to biotic and abiotic stresses than closely related wild species (Jena 2010; Nonomura et al. 2010). In addition, previous studies have shown genomic diversity with respect to genome size, genomic contents, and genomic polyploidy (Vaughan et al. 2003; Buell et al. 2005; Wing et al. 2005). These studies highlighted distantly related *Oryza* species as invaluable genetic and genomic resources for exploring and exploiting the hidden molecular mechanisms of agronomically important traits in breeding science and have made a good evolutionary

case for investigating the complex polyploidy problem in basic biology. In this NGS era, efforts to meet the challenge of deciphering distantly related wild *Oryza* genome sequences at a reasonable quality have been initiated. The achievements of several completed and ongoing projects thus far will be reviewed in the following sections.

1.11 Sequencing of *O. punctata* (BB) Genome

O. punctata, which belongs to the *Oryza officinalis* complex (Table 1.2), is a wild rice species distributed in Africa. The *Oryza officinalis* complex comprises diploid and tetraploid species with BB, CC, DD, and EE genomes. The DD genome has not yet been observed in a diploid state and has only been found in CCDD tetraploids. The species in the *O. officinalis* complex are geographically widely distributed in Asia (and the northern part of Australia), Africa, and South America. *O. punctata* accessions are categorized into two subtypes according to their genome types: diploids (BB) and tetraploids (BBCC), which grow in separate habitats. Their traits and genome sequences are of immediate interest, particularly to breeding scientists, since these species demonstrate resistance to multiple biotic and abiotic stresses (Jena 2010; Sanchez et al. 2013). The diploid BB *punctata* has $2n = 24$ chromosomes, and the nuclear genome size has been estimated as nearly equal to that of *O. sativa* (~400 Mb, Table 1.1) in flow cytometry experiments. An effort to decipher a diploid *punctata* (BB) accession genome has been made by Arizona Genomics Institute (AGI) under the activities of I-OMAP. A BAC-pooled WGS method using Illumina technology was undertaken, and the scaffolds were finally aligned to chromosomal coordinates according to the BAC-based physical map. As expected, the total assembly size was ~400 Mb, with a similar proportion of repetitive contents in the total genome to cultivated rice. This *O. punctata* genome sequencing has not yet been reported but in preparation, although the pre-publication assembly and baseline genome annotations have been released under the guidelines of the Fort Lauderdale Agreement (https://www.genome.gov/10506537/) in Ensembl Plants (http://plants.ensembl.org). From an evolutionary point of view, the availability of both BB and CC diploid genomes (see below) will be an epochal advance in the characterization of ancient genome-scale evolutionary events.

1.12 Sequencing of *O. officinalis* (CC) Genome

O. officinalis (Table 1.2) is a wild rice species that grows in various environments in South and Southeast Asia. Due to the wider habitats and putative core components of BBCC and CCDD tetraploid species, significant roles of *O. officinalis* in the

evolutionary history of rice have been speculated, whereas the relationship with other tetraploid species is not yet known. From a breeding perspective, similar to *O. punctata*, *O. officinalis* shows resistance traits related to multiple plant diseases (Jena 2010; Sanchez et al. 2013) and has been utilized as a genetic resource for introgression into cultivated rice species (Huang et al. 2001; Sanchez et al. 2013). However, there are limited genomic resources publicly available for this species. Consistent with other diploid species in the *Oryza* genus, this species comprises 2n = 24 chromosomes, but the nuclear genome size has been estimated as more than 600 Mb, which is one and a half times as large as those of AA species based on flow cytometry experiments (Uozu et al. 1997; Miyabayashi et al. 2007).

To serve as a quality genomic reference resource in breeding science to explore the biology of ploidy, the National Institute of Genetics of Japan has been promoting a nuclear genome sequencing project of an accession of *O. officinalis* under the collaborative activities of I-OMAP. A hybrid WGS approach using both Illumina and PacBio sequencing technologies has been adopted, and the resultant scaffolds have been aligned based on chromosomal coordinates according to the BAC-based physical map provided by AGI/I-OMAP. Baseline genome annotation and comparative genomic analysis have been conducted. A manuscript reporting these results is currently under preparation. The genomic reference of *O. officinalis* will be critical for providing a foundation for exploring the ploidy biology of BBCC, CC, CCDD, and unknown DD diploid species in the *O. officinalis* complex.

1.13 Sequencing of *O. brachyantha*, the Smallest *Oryza* Genome

O. brachyantha is a wild rice species distributed in tropical Africa and is located in the basal lineage in the phylogeny of the genus *Oryza* (Zou et al. 2008). The genome type of this species is FF, and the genome size is the smallest in the genus *Oryza*. This species exhibits many biotic- or abiotic-resistant traits, such as broad-spectrum resistance to rice bacterial leaf blight (Ram et al. 2010). Through a whole-genome shotgun sequencing approach using Illumina technology and BAC-end sequences generated via Sanger technology, sequence coverage of 104X was generated for *O. brachyantha* (Chen et al. 2013). The assembled sequence blocks were subsequently anchored to chromosomes using a cytogenetic approach, generating 12 chromosome sequences with a total of 261 Mb in size (Chen et al. 2013). The low activity of long terminal repeat (LTR) retrotransposons and high frequency of internal deletions of ancient long terminal repeat elements in the *O. brachyantha* genome led to its compact genome. Approximately 32,000 protein-coding genes were annotated in its genome, and only 70% of these genes are located in collinear positions compared with the *O. sativa* genome. These

nonlinear genes were enriched at pericentromeric or heterochromatic knobs compared with euchromatic regions in the *O. sativa* genome, resulting in a reduced level of gene collinearity in recombination-inert regions (Chen et al. 2013).

1.14 Perspectives

The rice reference genome has been contributing to the studies of a wide range of areas including plant physiology, molecular genetics, and breeding. It is expected that rice genomics will further extend to research on rice genetic diversity and genotype-phenotype interaction, such as quantitative trait loci analysis and GWAS for traits of agronomical importance. Since there should be a large number of unrecognized useful genes in *O. sativa* landraces as well as wild *Oryza* species, which were possibly lost in the modern cultivars including Nipponbare, comparative genomics approaches will discover such genes that can help create novel elite cultivars with beneficial traits.

It is interesting that known domestication-related traits of cereal crops were generally endowed through loss-of-function-type mutations or gene losses. Therefore, to detect large-scale indels, inversions, translocations, and present/absent variations of chromosomal segments, reference class complete genomes of a lot of accessions including wild species are envisaged. For this purpose, recently emerging ultra-long read sequencing technologies will be a key factor for future rice genomics. Moreover, development of computer algorithms to cope with such a gigantic dataset is an urgent issue. As novel experimental methods such as genome editing is quickly spreading, the next-generation breeding will be based on the combination of genome information analysis and such experimental technologies.

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Chapter 2
Small RNAs in Rice: Molecular Species and Their Functions

Yutaka Sato, Misuzu Nosaka-Takahashi, Toshiya Suzuki, and Sae Shimizu-Sato

Abstract Small RNAs are major components of gene regulatory pathways conserved among eukaryotes. In basic and applied sciences, RNA interference (RNAi) and artificial microRNAs (amiRNAs) are often used to modulate gene expression. The molecular mechanisms of RNAi are mainly studied in nematode or insect cells as models. Functional analyses of endogenous small RNAs, including studies of rice as a model, have greatly contributed to our understanding of plant biology. In plants, small RNA-based gene regulation has unique characteristics not found in animals, and many small RNAs regulate biological phenomena specific to plants. Recently, small RNA profiling using next-generation sequencers became possible, and various small RNA species were identified in plants including rice; their functional analyses are underway. This chapter summarizes the components of small RNA pathways, the molecular species of small RNAs, and the unique function of small RNAs in rice. It also considers the functions of small RNAs in relation to agriculturally important traits.

Keywords *Oryza sativa* · Rice · Small RNA · siRNA · miRNA · DICER · AGO

2.1 Introduction

Plant endogenous small RNA species (20–30 nucleotides (nt)) are classified mostly into microRNAs (miRNAs) and small interfering RNAs (siRNAs) on the basis of their precursor structures and processing (Table 2.1). miRNAs are produced from single-stranded RNAs with a stem–loop structure, whereas siRNAs are produced from double-stranded RNAs (dsRNAs) with nearly perfect complementarity. On

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Table 2.1 Classification of small RNAs in rice

	miRNAs		ta-siRNAs (phasiRNAs)		nat-siRNAs	hc-siRNAs
Precursor	Single-stranded RNAs		Double-stranded RNAs		Double-stranded RNAs	Double-stranded RNAs
Processing	DCL1	DCL3a	DCL4	DCL3b	DCL2	DCL3a
Length	21 nt	24 nt	21 nt	24 nt	24 nt	24 nt
Function	mRNA cleavage	DNA methylation	mRNA cleavage	DNA methylation	mRNA cleavage	DNA methylation
			DNA methylation			Chromatin remodeling

the basis of the differences in their production pathways, siRNAs are further classified into trans-acting siRNAs (ta-siRNAs), natural-antisense transcript-derived siRNAs (nat-siRNAs), and heterochromatic siRNAs (hc-siRNAs); the former group is sometimes described as a subset of phased-secondary siRNAs (phasiRNAs) based on the similarities in biogenesis pathways (Table 2.1) (Vaucheret 2006; Komiya 2017).

Since next-generation sequencers became available, small RNA profiles have been analyzed in various plant species allowing us to capture the landscape of small RNAs. Some endogenous small RNAs, such as a fraction of miRNAs (and their regulatory targets), are widely conserved among land plants, suggesting that small RNA-based gene regulation is essential for plant survival (see later).

Small RNA profiling in rice (Nobuta et al. 2007; Xue et al. 2009; Chen et al. 2011) has revealed that siRNAs are abundant, whereas miRNA content is low, although the ratio between the two types of small RNA differs among tissues analyzed. For example, of total small RNAs in developing rice seeds, 87.5% are siRNAs and only 0.5% are miRNAs (Fig. 2.1) (Xue et al. 2009). In these large-scale small RNA profiles, the same miRNA sequences are usually retrieved multiple times, whereas the same siRNA sequences are rarely retrieved. This means that each miRNA is expressed at a much higher level than each siRNA and that a tremendous number of different siRNAs are expressed in rice cells. Most rice siRNAs (71.9%) are derived from intergenic regions or repetitive sequences such as transposons. Because most repetitive regions are heterochromatic, siRNAs derived from these regions are hc-siRNAs and are believed to be involved in heterochromatin formation or maintenance. The proportion of nat-siRNAs among total siRNAs is very small (0.9%), whereas phasiRNAs become very abundant at the reproductive stage of rice development (Fig. 2.1).

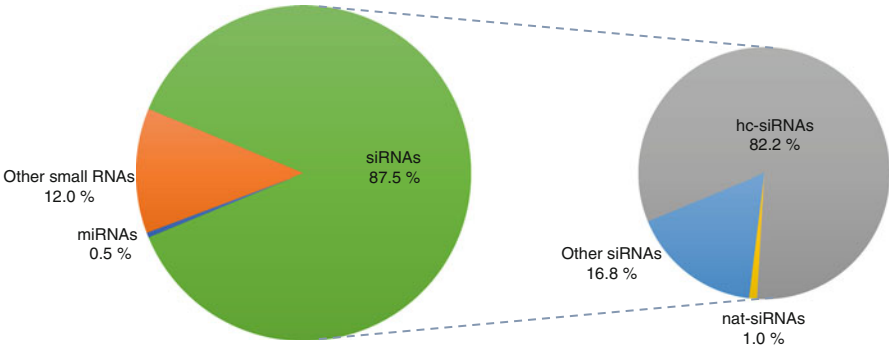

Fig. 2.1 Small RNA profiles in rice (Modified from Wu et al. 2010; Csorba et al. 2015; Borges and Martienssen 2015; Fang and Qi 2016; Komiya 2017). In developing rice seeds, 87.5% are siRNAs and only 0.5% are miRNAs. Among siRNAs, hc-siRNAs, nat-siRNAs, and other siRNAs compose 82.2%, 1.0%, and 16.8%, respectively

2.2 Protein Components of Small RNA Pathways in Rice

In plants, including rice, genes involved in miRNA or siRNA biogenesis or their targets often form families. For example, DICER, an enzyme required for generating small RNAs from fold-back structures of single- or double-stranded RNA, is encoded by four genes in *Arabidopsis* and five genes in rice (Margis et al. 2006; Kapoor et al. 2008). Rice DCL1 and DCL4/SHOOT ORGANIZATION1 (SHO1) produce 21-nt small RNAs, whereas DCL2, DCL3a, and DCL3b produce 22- or 24-nt small RNAs (Table 2.1). DCL1 is mainly engaged in miRNA biogenesis (Fig. 2.2a). Reduced DCL1 level results in developmental abnormalities possibly through decreased miRNA production (Liu et al. 2005). DCL2 produces nat-siRNA (Fig. 2.2d). DCL3a mainly produces hc-siRNA (Fig. 2.2e), and DCL3b produces phasiRNA (Fig. 2.2c) (Song et al. 2012). In rice, DCL3b is referred to as DCL5, a grass-specific DICER (Fei et al. 2013). In addition, DCL3a is involved in long miRNA (lmiRNA) processing (Fig. 2.2a); lmiRNAs are 24-nt miRNAs derived from potential stem–loop structures and act in a similar way to hc-siRNAs (Wu et al. 2010). DCL4 (SHO1) is required for generating ta-siRNA and phasiRNA (Fig. 2.2b, c) (Nagasaki et al. 2007; Liu et al. 2007; Song et al. 2012). Loss-of-function of DCL4 (SHO1) results in defective embryo development due to abnormal shoot organization (Nagasaki et al. 2007). DCL4 knockdown results in abnormal spikelets (Liu et al. 2007).

Small RNAs produced by DICERs are incorporated into Argonaute (AGO) proteins, which are a major component of the RNA-induced silencing complex (RISC); AGOs work as effectors of gene regulation. The rice genome contains 19 AGO copies, and the *Arabidopsis* genome has 10 copies (Kapoor et al. 2008).

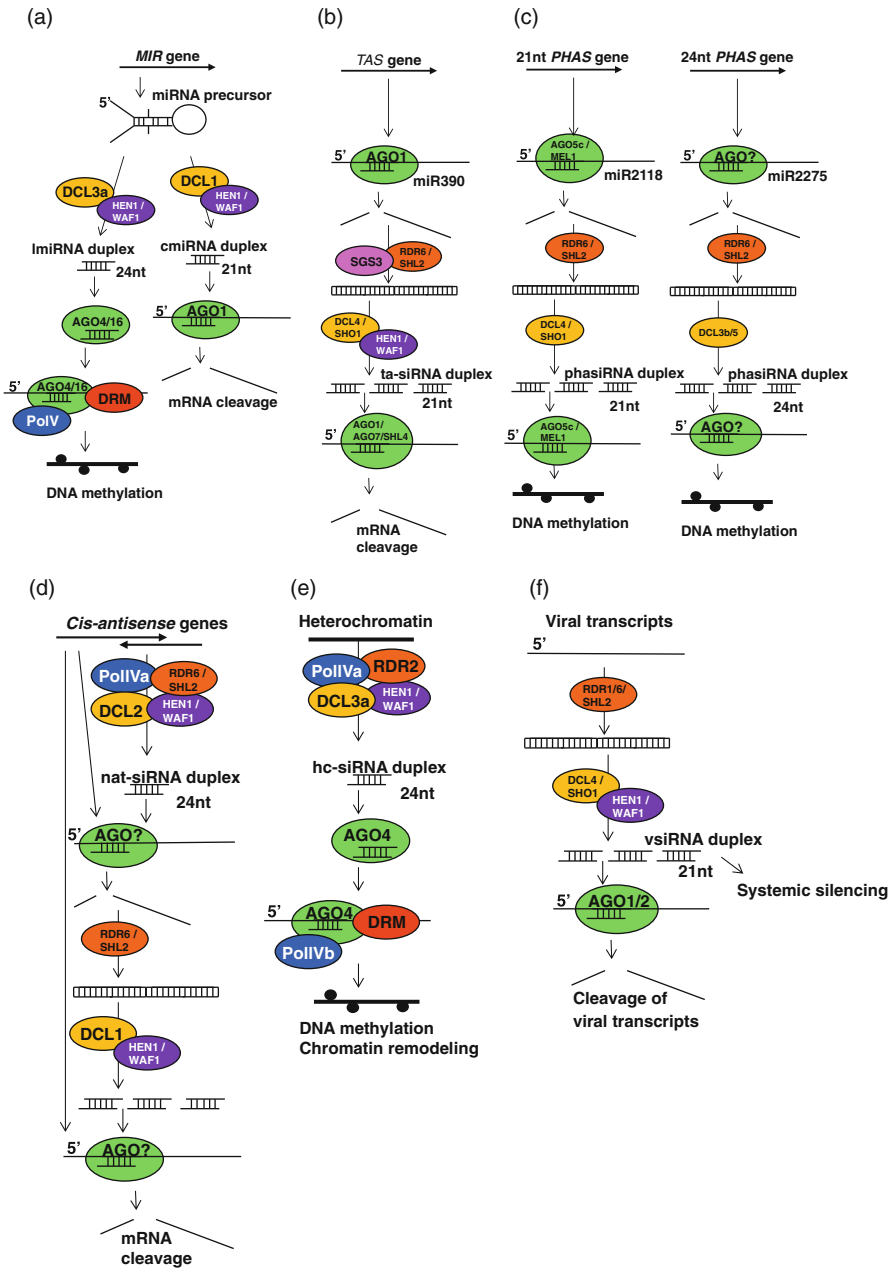

Fig. 2.2 Small RNA pathways in rice (Modified from Nobuta et al. 2007; Xue et al. 2009; Chen et al. 2011). (**a**) microRNA (miRNA) pathway. (**b**) Trans-acting siRNA (ta-siRNA) pathway. (**c**) Phased-secondary siRNA (phasiRNA) pathway. (**d**) Natural-antisense transcript-derived siRNA (nat-siRNA) pathway. (**e**) Heterochromatic siRNA (hc-siRNA) pathway. (**f**) Viral siRNA (vsiRNA) pathway

The base at the 5′ terminus of a small RNA determines AGO selectivity (Mourrain et al. 2000; Takeda et al. 2008), but selective incorporation of small RNAs alone does not explain the functional diversification of rice AGOs. For example, rice *SHOOTLESS4* (SHL4) encodes an ortholog of *Arabidopsis AGO7* (Nagasaki et al. 2007). Analysis of a *shl4* mutation revealed that *SHL4* is required for the regulation of *ETTIN* transcription factor genes by ta-siARF, one of the specific ta-siRNAs. *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*) in rice also encodes an AGO and is expressed in a cell type-specific manner; the phenotype of a mutation in this gene is observed in meiosis (Nonomura et al. 2007). Thus, the functional diversification of these AGOs is not related to biogenesis of small RNAs or the 5′ base of small RNAs but rather relies on their cell type-specific or developmentally regulated expression.

RNA-DEPENDENT RNA POLYMERASEs (RDRs) are required for dsRNA production. dsRNAs are substrates of DICER, which cleaves them into 21- or 24-nt small RNAs. The rice genome encodes six RDRs (RDR1–RDR6/SHOOTLESS2 (SHL2)); their precise roles are not well documented except for RDR2 and RDR6/SHL2. RDR2 produces hc-siRNA, whereas RDR6/SHL2 seems to have a broader function. RDR6/SHL2 generates viral double-stranded RNAs and is important for defense against RNA viruses (Jiang et al. 2012) and is also essential for rice development (Nagasaki et al. 2007; Toriba et al. 2010). Its loss-of-function *shootless2* (*shl2*) mutation leads to a severe phenotype during embryogenesis (the lack of the shoot apical meristem region). Weak alleles of *RDR6*/*SHL2* show abnormal spikelet development, which is mostly explained by the loss of ta-siARF. Thus, *RDR6*/*SHL2* is required for producing double-stranded RNA for ta-siRNA.

Arabidopsis HUA ENHANCER1 (HEN1) transfers the methyl group to the 3′-end hydroxyl group of small RNAs and stabilizes them. Rice *WAVY LEAF1* (*WAF1*) is an ortholog of *HEN1*; the *waf1* mutation causes severe developmental abnormalities (Abe et al. 2010). *Arabidopsis* small RNA pathways also include the SUPPRESSOR OF GENE SILENCING3 (SGS3), HASTY, HYPONASTIC LEAF1 (HYL1), and SERRATE proteins, and their loss-of-function mutants revealed their functions (Han et al. 2004; Borges and Martienssen 2015), but there is no clear description of the function of these components in rice. Considering the conservation of function and components of small RNA pathways in plants, these proteins probably function similarly in rice (Fig. 2.2). Overall, most genes working in small RNA pathways in rice were originally found from mutations with developmental abnormality such as embryogenesis-defective mutants. In addition, heterochronic mutation that affects juvenile to adult transition in *Arabidopsis*, *Maize*, and rice also helped to realize small RNA pathways and the function of several miRNAs, such as miR156 and miR172 (Auckerman and Sakai 2003; Hunter et al. 2003; Peragine et al. 2004; Lauter et al. 2005; Wu and Poething 2006; Smith et al. 2009; Chuck et al. 2007; Tanaka et al. 2011; Yoshikawa et al. 2013; Hibara et al. 2016). Thus, further analyses of these mutations would enforce our understanding on small RNA pathways in rice.

2.3 miRNA Gene Annotations in Rice

Sequences of mature plant miRNAs and their precursors are deposited in miRBase (http://www.mirbase.org). Each miRNA is numbered, and rice miRNAs are marked by letters representing the species name, for example, osa-miR156. The use of the same identifying number in different plant species means that the miRNA is conserved. In plants, the same miRNA is often produced from multiple genetic loci that form gene families. The primary transcript of each family member is marked by a lowercase letter, for example, osa-miR156a.

Among rice miRNA sequences deposited to miRBase, osa-miR156, 319/159, 160, 166, 171, 390, and 408 are the most conserved across land plants. They are followed by osa-miR162, 164, 167–169, 172, 393–395, 397–399, and 827, which are conserved in gymnosperms and angiosperms. The rest of rice miRNAs are specific to monocots, grasses, or rice. Usually, conserved miRNAs share the same target genes, which are also conserved (Cuperus et al. 2011).

In miRBase release 21, there are 575 entries of genomic loci for rice miRNA genes. Not all rice miRNA gene loci in the database produce mature miRNA, but their precursor transcripts potentially form stem–loop structures, and corresponding mature miRNA sequences have been present in several RNA profiles obtained by next-generation sequencing. The miRbase entries of rice miRNA genes could potentially contain 330 different mature miRNAs. In rice, miR395 forms the largest family with 25 members, followed by osa-miR812 (19 members), osa-miR169 (18), osa-miR2118 (18), osa-miR1861 (14), osa-miR166 (13), osa-miR156 (12), osa-miR399 (11), and osa-miR167 (10); some other families contain fewer than 10 members. Most of the conserved miRNAs form gene families; among conserved miRNAs listed above, only osa-miR390, 408, 394, and 827 are encoded by single-copy genes.

Unfortunately, the annotations of miRNA genes in the MSU database (http://rice.plantbiology.msu.edu) and RAP-DB (http://rapdb.dna.affrc.go.jp) are incomplete. For example, only 134 (MSU) and 170 (RAP-DB) gene IDs are assigned to the stem–loop regions of the 575 rice miRBase entries. The conserved miRNAs listed above are most likely functional and could be produced from 136 gene families, but only 14 (MSU) and 44 (RAP-DB) gene annotation IDs are assigned. Recent mRNA-seq analysis using next-generation sequencers clearly faces difficulties in detecting changes in the expression of most miRNA genes.

2.4 Small RNAs and Abiotic Stress Responses in Rice

Rice miRNAs and siRNAs play important roles in responses and tolerance to abiotic stresses (Jeong and Green 2013). Deficiency in inorganic phosphate (Pi) frequently limits plant growth and development. The relationship between rice miRNAs and phosphorus (Pi) deprivation response is well studied. Expression

of rice osa-miR399 is strongly induced by Pi starvation (Bari et al. 2006). Its target gene, *LEAF TIP NECROSIS1* (*LTN1*), was originally isolated from rice plants with mutations in this gene (Hu et al. 2011). *LTN1* is a putative ortholog of *Arabidopsis PHO2*, which encodes a ubiquitin-conjugating E2 enzyme (UBC24) and is important in Pi starvation signaling (Aung et al. 2006). Overexpressed osa-miR399 downregulates *LTN1* by degrading its transcript and increases Pi accumulation. Whereas osa-miR399 signaling is conserved between rice and *Arabidopsis*, osa-miR827 seems to regulate the Pi starvation response differently in these species. In *Arabidopsis*, the target gene of miR827 is *NLA* (*Nitrogen Limitation Adaptation*), which encodes a RING-type ubiquitin ligase with an SPX domain (SYG1/Pho81/XPR1); proteins containing this domain participate in Pi transport or Pi sensing. In *Arabidopsis*, the expression of miR827 increases in response to both nitrogen and Pi deficiency (Peng et al. 2007). In rice, osa-miR827 is upregulated by Pi starvation only (Lin et al. 2010). Analysis of transgenic plants and knockout lines indicates that rice osa-miR827 downregulates *OsSPX-MFS1* and *OsSPX-MFS2*, which encode proteins containing both an SPX domain and a major facilitator superfamily (MFS) domain; the MFS domain is found in membrane proteins involved in small solute transport. The level of *OsSPX-MFS1* mRNA is reduced by Pi starvation possibly because of the upregulation of osa-miR827, but that of *OsSPX-MFS2* mRNA is increased, suggesting a complex regulation of *OsSPX-MFS1* and *OsSPX-MFS2* in response to Pi starvation.

Comprehensive large-scale analyses by deep sequencing have revealed miRNAs related to Pi homeostasis in rice (Secco et al. 2013). Under Pi starvation, both osa-miR399 and osa-miR827 are upregulated. Twenty other miRNAs have been newly identified as Pi starvation responsive; 80% of these have been found only in rice. Among the latter miRNAs, the expression level of osa-miR3979 was dramatically reduced by Pi starvation, although osa-miR3979 was also downregulated by nitrogen starvation (Jeong et al. 2011). osa-miR444 was also induced not only by Pi starvation but also by nitrogen starvation (Yan et al. 2014). These results suggest that the network of rice miRNAs cooperatively signal in response to nutrient deprivation, promoting survival under severe stress conditions.

Drought and salt stresses are the most severe abiotic stresses and are also a major agricultural problem. osa-miR393 is induced by drought stress (Zhao et al. 2007) and targets two auxin receptor genes, *OsTIR1* (*Transport Inhibitor Response Protein 1*) and *OsAFB2* (*Auxin Signaling F-box Protein 2*) (Xia et al. 2012). Transgenic rice overexpressing osa-miR393 shows a reduced auxin response and reduced tolerance to drought and salt. The function of osa-miR156 is related to abiotic stress and rice development (Cui et al. 2014). osa-miR156 is strongly induced by drought and salt stresses (Jeong et al. 2011), and its overexpression increases stress tolerance. This miRNA also influences the metabolism of anthocyanin, which is accumulated in response to various stresses including drought and salt, through *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9* (*SPL9*) and its downstream gene, *DIHYDROFLAVONOL-4-REDUCTASE* (*DFR*).

Rice miRNAs are also induced by cold, heat, and oxidative stresses and by nonessential heavy metals. osa-miR319 is downregulated by cold stress, and its

overexpression increases cold tolerance by downregulating its target genes, *Os PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR 6* (*OsPCF6*) and *Os TEOSINTE BRANCHED/CYCLOIDEA/PCF* (*OsTCP21*), which are the plant-specific transcription factors containing bHLH motifs that allow DNA binding and protein–protein interaction (Wang et al. 2014). Overexpression of osa-miR529 increases rice tolerance to high levels of H_2O_2 (Yue et al. 2017). Overexpression of osa-miR390 increases Cd accumulation and reduces Cd tolerance (Ding et al. 2016). Transgenic rice plants overexpressing these miRNAs have an increased or decreased tolerance to abiotic stresses and nutrient deprivation. Further analysis of the molecular mechanisms of small RNA-mediated gene regulation in response to environmental stresses is expected to contribute to increasing yield and quality in crop production.

2.5 Small RNAs and Immune Response in Rice

Plants, including rice, are attacked by pathogenic bacteria, fungi, and viruses. As the first step of the immune response, conserved pathogen-associated molecular patterns (PAMPs) are recognized by the host and elicit PAMP-triggered immunity (PTI) to limit pathogen propagation. However, some pathogens produce effector proteins that suppress PTI. To counteract such pathogens, plants recognize the effector proteins with their resistance (R) proteins and activate the second step of the immune response, called effector-triggered immunity (ETI). In rice, both PTI and ETI respond to bacterial and fungal pathogens and lead to resistance. Small RNAs have an important role in rice immune responses, as outlined below (Seo et al. 2013; Baldrich and San Segundo 2016).

2.5.1 Small RNAs in Rice–Bacterium Interactions

The main bacterial disease of rice worldwide is bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In cultivated rice, the major disease resistance gene, *Xa3*/*Xa26*, encodes a leucine-rich repeat kinase-like protein (Sun et al. 2004). Because *Xa3*/*Xa26* alleles in wild rice species (BB and BBCC genomes) also confer resistance to *Xoo* (Li et al. 2012), *Xa3*/*Xa26*-mediated resistance is durable. To investigate the role of small RNAs in *Xa3*/*Xa26*-mediated resistance, simultaneous genome-wide analyses of the expression of small RNAs and genes of rice inoculated with *Xoo* was carried out (Hong et al. 2015a). A number of miRNAs, some siRNAs, and a large number of genes were differentially expressed in plants with *Xa3*/*Xa26*-mediated resistance. Genes encoding the receptor-like kinase OsWAK and a UDP-glucosyltransferase domain-containing protein are likely regulated by small RNAs and involved in resistance.

2.5.2 Small RNAs Involved in Rice Immunity Against Blast Fungus

Rice blast is the most devastating disease affecting rice production. Rice blast fungus, *Magnaporthe oryzae*, can infect rice plants at any developmental stage. Overexpressed osa-miR7695 promotes rice resistance to *M. oryzae* by suppressing a metal transporter gene (Campo et al. 2013). Overexpressed osa-miR160a or osa-miR398 also enhances rice resistance to *M. oryzae*, which is associated with increased H_2O_2 accumulation at the infection site and upregulation of the expression of defense-related genes (Li et al. 2014). osa-miR398 is likely involved in PTI signaling, and osa-miR160 may be involved in ETI signaling.

Deep sequencing of small RNA libraries for global identification of rice miRNAs that are regulated by an elicitor produced by *M. oryzae* revealed that some of these miRNAs regulate genes which act in a small RNA pathway (miR393 regulates SGS3, miR168 regulates AGO1), hormone signaling, and a cross talk among various hormone pathways (Baldrich et al. 2015). The expression of miRNA target genes classified as "signaling" increases and that of genes classified as "development" decreases in elicitor-treated rice. Thus, it seems that miRNAs regulate the growth–defense balance. Of interest, the rice-specific osa-miR5819, osa-miR5075, and osa-miR2101 target the conserved peptide upstream open reading frame (CPuORF)-containing genes encoding CPuORF3-OsbZIP38, CPuORF4-OsbZIP27, and CPuORF7-SAM decarboxylase, respectively. CPuORF3-OsbZIP38 and CPuORF4-OsbZIP27 contain a short version of the sucrose control-upstream open reading frame (SC-uORF). This observation suggests a connection between small RNAs and sucrose-regulated translational control of the expression of these two rice bZIP transcription factors. This regulatory network integrates two of miRNA functions; one in metabolic regulation of gene expression and the other in rice immune responses.

2.5.3 Small RNAs in Rice–Virus Interactions

One of the major functions of the small RNA pathway is the defense against genomic parasites such as RNA viruses or transposons (Nosaka et al. 2012; Csorba et al. 2015). Rice virus diseases are generally restricted to specific rice-growing areas. *Rice stripe virus* (RSV) and *Rice dwarf virus* (RDV) are found in Asia. RSV is a negative-sense single-stranded RNA virus, and RDV is a double-stranded RNA virus. *Rice black-streaked dwarf virus* (RBSDV), a double-stranded RNA virus, infects various cereal species, including rice and maize. Small RNA profiling of rice plants infected by RDV or RSV demonstrated distinct effects of these viruses on the small RNA population (Du et al. 2011). RSV but not RDV infection interferes with the miRNA pathway and enhances the accumulation of some rice miRNA*s which are the opposite strands of miRNAs and are usually less stable than miRNAs. The

asterisk after miRNA denotes the opposite strand of miRNA. Rice RDR6 functions in the defense against RSV, whereas RDV infection downregulates RDR6 expression. Silencing of RDR6 enhances rice susceptibility to both RSV and RDV infections (Jiang et al. 2012; Hong et al. 2015b). However, overexpressed RDR6 does not improve resistance to RDV infection (Hong et al. 2015b). RDR6 protein accumulation in RDR6-overexpressing lines clearly decreases after RDV infection, possibly because of translational suppression of RDR6 and/or destabilization of the protein by RDV. Thus, there is a cross talk between the host and viruses.

2.5.4 Application of miRNA-Based Strategies to Produce Disease-Resistant Rice

Several disease-resistant transgenic rice lines have been produced by overexpressing either osa-miR160, osa-miR398, or osa-miR7695 against fungi and by overexpressing osa-miR444 or knocking out osa-miR528 to protect against viruses (Baldrich and San Segundo 2016; Wang et al. 2016; Wu et al. 2017). Artificial microRNAs, miRNA target mimicry, TALEN, and CRISPR/Cas9 technologies are also applicable for the improvement of disease resistance. Therefore, it is important to know the functions of small RNAs and small RNA pathways, as this knowledge could be applied to developing new methods of disease control in rice.

2.6 Rice Small RNAs and Crop Production

High-throughput sequencing revealed that diverse small RNAs including more than 300 miRNAs and various numbers of 24-nt siRNAs are expressed in rice grains and spikelets (Peng et al. 2011, 2013). They are predicted to be involved in various metabolic and developmental pathways, and they and their target genes could be used for increasing rice crop yield.

Several rice miRNAs affect grain productivity. Two groups independently identified a QTL named *IPA1*/*WFP*, which increases the number of panicle branches (Jiao et al. 2010; Miura et al. 2010). The *IPA1*/*WFP* locus encodes the plant-specific transcription factor SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14 (OsSPL14), which is regulated by osa-miR156 and osa-miR529. Increased expression of *OsSPL14* in panicles caused by osa-miR156 increases grain production (Jiao et al. 2010; Miura et al. 2010). Introduction of the $OsSPL14^{WFP}$ allele into the standard *Japonica* cultivar Nipponbare increased the number of panicle branches and grain production (Miura et al. 2010). Another *Japonica* cultivar, Aikawa 1, has a single nucleotide change at the osa-miR156-

targeted site in *OsSPL14*. Both the expression level of *OsSPL14* in young panicles and the number of panicle branches are higher in Aikawa 1 than in Nipponbare (Miura et al. 2010). Although the number of panicle branches was reduced in transgenic plants overexpressing osa-miR156 and osa-miR529 or in *OsSPL14* RNAi lines, overexpression of *OsSPL14* had a similar effect (Xie et al. 2006; Wang et al. 2015). This indicates that fine-tuning of *OsSPL14* expression is important for increasing rice grain yield as exemplified by $OsSPL14^{WFP}$ allele introduction (Miura et al. 2010). osa-miR172 targets five AP2-like transcription factors (*SNB*, *OsIDS1*, *SHAT1*, *OsTOE1*, and *OSGL15*) and is involved in panicle branching. Inhibition of osa-miR172 activity or overexpression of *SUPERNUMERARY BRACT* (*SNB)* and *OsTARGET OF EARLY ACTIVATION TAGGED1* (*OsTOE1*) causes dense panicles with a significantly larger number of branches and spikelets than in the wild type (Wang et al. 2015). The fact that OsSPL14 directly regulates osa-miR172 expression indicates the cooperative function of osa-miR156, osa-miR529, and osa-miR172 in reproductive branching (Wang et al. 2015). Overexpressed osa-miR397 enlarges grains and promotes panicle branching, thus increasing grain yield by up to 25% (Zang et al. 2013). This increase is due to downregulation of its target *OsLAC*, which encodes a laccase-like protein involved in sensitivity to brassinosteroids. Blocking osa-miR396 activity greatly increases grain yield by increasing the number of panicle branches and spikelets through a direct induction of *GROWTH-REGULATING FACTOR 6* (*OsGRF6*) (Gao et al. 2015). Another target of osa-miR396 is *OsGRF4*, which controls grain size and weight (Duan et al. 2015). Therefore, osa-miR396 controls both panicle branching and grain size. osa-miR398 targets Cu/Zn-superoxide dismutase genes and is also a promising target for improvement of rice grain yield (Zhang et al. 2017).

Grain productivity in rice is also affected by siRNAs. The RNAi lines of rice *DCL3a*, which produce 24-nt siRNAs associated with miniature inverted-repeat transposable elements, have fewer panicle branches and show other phenotypic defects compared with wild type (Wei et al. 2014). These siRNAs target many gibberellin and brassinosteroid homeostasis-related genes, indicating that the siRNAs are involved in panicle branching, which is regulated by these hormones.

Heterosis, the superior performance of hybrids in comparison with their parents, is an important phenomenon that helps to improve crop production. Some groups found differential expression of rice small RNAs between hybrids and their parents by using high-throughput sequencing (He et al. 2010; Chen et al. 2010; Zhang et al. 2014); this suggests that small RNAs have a potential role in rice hybrid vigor. Target genes of some differentially expressed small RNAs encode auxin response factors (ARFs) and some transcription factors (NAC1, PHAVOLUTA, and REVOLUTA) involved in the auxin signaling pathway (Zhang et al. 2014).

2.7 Conclusion

Recent progress in small RNA sequencing in plant cells has revealed a higher diversity of the molecular species of small RNAs than that of protein-coding mRNAs and opened a new paradigm in gene regulation. Only a limited number of small RNA functions in rice have been described so far. hc-siRNAs function in epigenetic modifications of DNA; the details are described in another chapter of this book (Chap. 24). Although the functions of most small RNAs are not yet elucidated and new modes of gene regulation by small RNAs may be found in the future, small RNAs and their regulation in rice are potentially useful for improving crop production.

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Chapter 3
Composition and Structure of Rice Centromeres and Telomeres

Hiroshi Mizuno, Takashi Matsumoto, and Jianzhong Wu

Abstract Complete or partial sequences of centromeres and telomeres of a number of rice chromosomes are now publicly available. In this chapter, we summarize the current findings on the DNA content and structure of these special regions. Core regions of rice centromeres consist of the satellite repeat CentO organized in tandem arrays and copies of the Ty3/*gypsy*-type retrotransposon CRR physically associated with the CentO arrays. The physical size of CentO arrays differs considerably among individual chromosomes. Unexpectedly, rice centromeres also contain active genes. Telomere satellite repeat arrays are highly conserved within the plant kingdom, but their length varies considerably among species and even among rice chromosomes. Frequent nucleotide substitutions and rearrangements of the satellite repeat sequences are detected mainly within the proximal telomere regions in rice. Subtelomere-associated repeats appear to be species-specific; at least 14 of its 24 chromosomal ends in the rice cultivar Nipponbare contain no TrsA sequences, that is, the telomere arrays are directly attached to gene-containing regions. Despite the conservation of functions, both centromeres and telomeres in rice reveal a considerable size variation and sequence divergence, thereby providing insights into the structural and evolutionary dynamics of these highly heterochromatic regions.

Keywords Rice · Centromere · Telomere · Satellite repeat array · Retrotransposon

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3.1 Introduction

Eukaryotic chromosomes usually consist of chromosome arms, the centromere, and telomeres. Located between the two arms of each chromosome, the centromere serves as a place of attachment for spindle fibers during cell division which mediates faithful chromosome segregation to ensure that each new daughter cell has the correct number of chromosomes after mitosis and meiosis, whereas the telomere at each chromosome end is a region which is essential for maintaining chromosomal and genomic stability to protect from enzymatic end-degradation or from fusion with neighboring chromosomes (Fig. 3.1). Knowing sequence composition and organization within these regions is important for understanding their function. Rice is one of the most important cereal crops, and the entire genome sequence of the Asian rice cultivar Nipponbare (*Oryza sativa* L. ssp. *japonica*) has been completely deciphered and annotated by the International Rice Genome Sequencing Project (IRGSP) (2005) and Kawahara et al. (2013). The release of this map-based high-quality sequence facilitates research on the genetic, biological, evolutionary, and functional aspects of the rice genome and will increase the efficiency of molecular breeding of this economically important crop (Han et al. 2007; Wu et al. 2008; Zuo and Li 2013; Matsumoto et al. 2016).

3.2 Rice Centromere

Similar to other higher eukaryotes such as animals (Schueler et al. 2001), plants have a centromeric region on each chromosome, which consists of several megabases (Mb) of highly repetitive DNA sequences (Dong et al. 1998; Richards and Dawe 1998; Copenhaver et al. 1999). Because a clone-by-clone sequencing

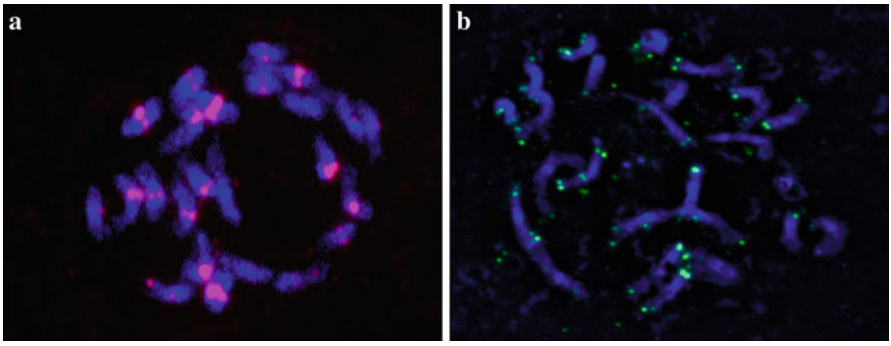

Fig. 3.1 FISH analysis of chromosomes from the *japonica* rice cultivar Nipponbare (*O. sativa* **L**, 2n=24). Probes of CentO and TTTAGGG repeats hybridize exclusively to the centromeres (**a**, red) and telomeres (**b**, green), respectively. DAPI (4′,6-diamidino-2-phenylindole) stained chromosomes are shown in blue

strategy is adopted by the IRGSP, the sequence of the Nipponbare genome entirely or partially covers the centromeres of all 12 chromosomes, making rice an excellent model for extensive studies of plant centromere structure, evolution, and function (Lamb et al. 2004; Hall et al. 2004; Ma et al. 2007a; Yan and Jiang 2007).

3.2.1 *Genetically and Physically Mapped Centromere*

A high-density rice linkage map indicates a strong reduction of recombination frequency around all centromeres (Harushima et al. 1998). Localization of DNA markers including centromere-specific repetitive sequences from the linkage map on the physical maps of the rice genome based on yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs) or P1-derived artificial chromosomes (PACs) identified the recombination-suppressed centromere region on all rice chromosomes (Chen et al. 2002; Wu et al. 2002, 2003). Integration of the latest genetic map with the current genome sequence map (IRGSP-1.0) has determined the physical size of the above genetically mapped centromere region (lacking genetic recombination) on each rice chromosome (Fig. 3.2; Table 3.1), ranging from 1.3 Mb on chromosome 12 (Chr12) to 5.5 Mb on chromosome 6 (Chr6); the total length of the centromeres reaches 37.3 Mb, which is nearly 10% of the entire genome (~386.5 Mb).

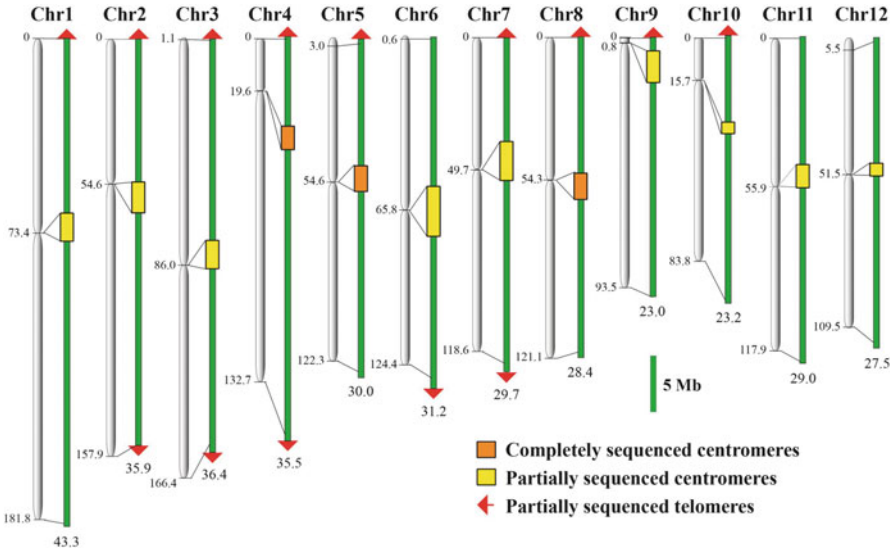

Fig. 3.2 Chromosomal position and sequencing status of centromeres and telomeres of Nipponbare rice. The pseudomolecule (IRGSP-1.0) for each chromosome (green) is shown according to its length (Mb; indicated at the bottom), and the corresponding genetic map (cM) is shown in gray on the left

Table 3.1 Chromosomal localization and physical size of genetically mapped centromeric regions in rice

Chr No.	Flanking marker	Position on genetic map (cM)	Position on sequence map (bp)	Length (kb)[a]
1	Y6855RB-S3382S	73.4	15,445,668–18,052,668	4025
2	C900-E1107S	54.6	12,625,206–15,485,898	3578
3	L708-R3235	86.0	17,883,934–20,517,434	2816
4	G124B-C954	19.6	7,882,030–10,065,097	2183
5	C288A-R3069	54.6	11,158,997–13,544,591	2386
6	C62815S-R688	65.8	13,201,849–17,840,759	5452
7	G1068-C479	49.7	9,104,733–12,717,407	3932
8	C1374-R2466	54.3	11,984,652–14,418,992	2434
9	E31165S-C3096SA	0.8	993,696–3,930,561	3552
10	C8-C489	15.7	7,624,447–8,696,452	1543
11	C53961-SE61044S	55.9	11,347,881–13,506,686	4055
12	C625-C443	51.5	11,061,326–12,242,574	1337

[a]Values calculated on the basis of both the genomic sequence and the FISH analysis of CentO contents (Cheng et al. 2002)

3.2.2 Composition and Structure of Centromere

Plant heterochromatin harbors abundant heterogeneous sequences, mainly transposable elements and tandem repeat arrays. Molecular and cytological studies have found that all rice centromeres carry the DNA sequences of a 155-bp satellite repeat CentO and a centromere-specific retrotransposon CRR (Dong et al. 1998; Miller et al. 1998; Nonomura and Kurata 1999, 2001). Varying in an amount from 64 kilobases (kb) to 1.9 Mb (as determined by fluorescence in situ hybridization, FISH), the CentO sequences in each centromere are confirmed particularly to locate within genomic regions to which the spindle fibers attach, and are physically coupled with CRRs, implying that CentO satellite repeats are key DNA elements for the formation and function of centromeres in rice (Cheng et al. 2002).

Satellite Repeats Rice is the only multicellular eukaryote to have three centromeres [those of Chr4 (Zhang et al. 2004), Chr5 (Cheng et al. 2005; Mizuno et al. 2011), and Chr8 (Nagaki et al. 2004; Wu et al. 2004)] completely sequenced. The current genomic sequence (IRGSP-1.0) of Nipponbare contains about 7100 copies (including >6900 copies with intact or almost intact sequences) of the CentO satellite repeat assembled within the centromeric regions of all chromosomes (Table 3.2). These copies (about 1133 kb in total) can be grouped into two distinct subfamilies with a consensus length of 155 bp (3358 copies) and 165 bp (2816 copies); the only difference between these subfamilies is a single 10-bp insertion. It is worth noting that no CentO copies on Chr8 belong to the 165-bp subfamily,

Table 3.2 Centromere- and telomere-specific satellite repeat sequences detected in the pseudomolecule sequence of the Nipponbare genome (IRGSP-1.0)

	Centromere					Telomere	
		Copy number of CentO repeat		Number of CentO tracts	Amount of CentO sequence (kb)	Copy number of TTTAGGG repeat	
Chr	Physical position (bp)	Short arm	Long arm			Short arm	Long arm
1	16,700,494–17,133,976	521	477	24	156.0	16	–
2	13,570,918–13,757,457	506	361	10	144.3	50	68
3	19,541,141–19,652,297	15	179	10	33.6	17	73
4	9,756,387–9,880,716	362[a]		20	56.6	127	52
5	12,458,306–12,555,474	543[a]		2	84.9	52	–
6	15,424,583–15,490,548	No	398	2	61.6	–	36
7	11,960,905–12,281,277	445	565	14	162.8	13	122
8	12,920,575–13,840,293	451[a]		6	69.7	53	–
9	2,750,494–2,981,252	234	547	5	124.7	153[b]	–
10	8,099,672–8,178,475	272	5	10	44.3	60	–
11	12,045,466–12,329,498	91	416	11	78.8	–	–
12	11,772,304–12,072,846	424	310	14	115.3	–	–

[a]Completely sequenced centromeres

[b]Calculated from the genomic sequences of the fosmid clones, OSJNOa109J19 (AP008225) and OSJNOa063K24 (AP009051) (Fujisawa et al. 2006), which are currently not included in IRGSP-1.0

whereas about 34% (121 copies) of all CentO copies on Chr4 and 13% (81 copies) on Chr5 belong to the 165-bp subfamily. Sequence similarity is higher between CentO repeats within the same centromere than between those from different centromeres, reflecting local homogenization of satellite sequences (Lamb et al. 2007; Macas et al. 2010; Yan et al. 2006). Only 20 CentO copies were detected in the regions outside genetically defined centromeres.

Transposable Elements The centromere region (~2 Mb) on rice Chr8 contains >220 transposable element-related sequences (Wu et al. 2004). Most of them belong to the Ty3/*gypsy*-type retrotransposon family and include such elements as hopi, RIRE3, and RIRE8, which are also present outside the centromeres. The Ty3/*gypsy*-type retrotransposons also include 29 full-size, truncated, or degenerated CRR sequences and a few of its solo long terminal repeats (LTRs). Most centromeric CRR elements are located within or flank the CentO satellite repeats. Similar results have been reported for the fully sequenced centromeres of Chr4 and Chr5 and partially sequenced centromeres of all other rice chromosomes (Yan et al. 2006; Jiang 2013; Nagaki 2009), confirming the involvement of CRR and CentO satellite sequences in the formation of the centromere core domain in rice. The CRR elements from multiple chromosomes have been grouped into four

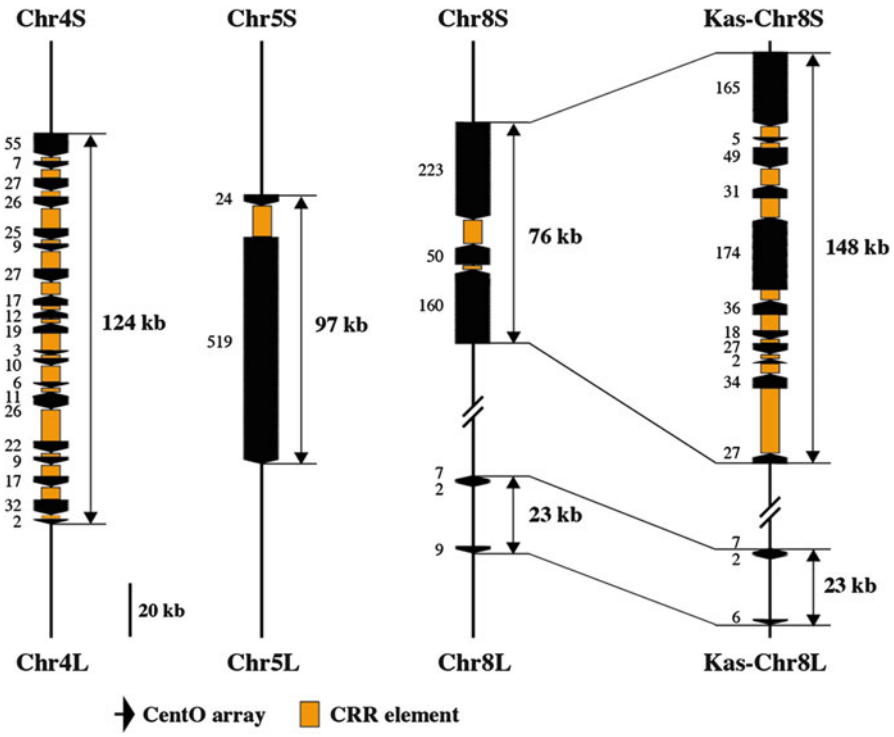

Fig. 3.3 Genomic composition and structure of CentO regions in the centromeres of the rice cultivars Nipponbare and Kasalath (Kas). Physical length (right) of each CentO cluster and the copy number (left) of satellite repeat within each CentO tract of chromosomes are shown

subfamilies by phylogenetic analysis: autonomous CRR1, autonomous CRR2, nonautonomous CRR1 (noaCRR1), and nonautonomous CRR2 (noaCRR2); noaCRR1 elements are more frequent in centromeres than are noaCRR2 elements (Nagaki et al. 2005).

CentO Core Organization Repetitive sequences occupy ~60% of the centromere on rice Chr8 (Wu et al. 2004). The core region in the center of each centromere is formed by tandemly arrayed head to tail CentO satellite repeat sequences which are organized into clusters of tracts with different orientations and copy number and are intermingled with CRR elements (Fig. 3.3). The CentO cluster in the centromere of Chr4 contains 20 CentO tracts (362 copies of the satellite repeat, 57 kb) (Table 3.2), which are accompanied by CRR elements to span a region of about 124 kb. On Chr5, the CentO cluster consists of only two CentO tracts (24 and 519 copies, 85 kb in total) interrupted by one CRR element within a 97-kb genomic region. On Chr8, three (433 copies, 67.1 kb) of the six CentO tracts form a large cluster (76 kb) in the center of the centromere. The remaining three short tracts constitute a small cluster carrying only 2.6 kb of CentO satellite sequences and located proximal to the long arm about 820 kb from the large cluster. Unlike in the large cluster, the CentO tracts

within the small cluster are interrupted by sequences derived from the non-centromere-specific Ty3/*gypsy*-type retrotransposon RIRE8. CentO satellite repeat sequences are less disturbed by CRR elements on Chr5 and Chr8 than on Chr4 in the Nipponbare genome (Fig. 3.3), indicating that the CentO cores of the centromeres of Chr5 and Chr8 are relatively compact. Because of the presence of a large number of satellite repeats, only partial sequences of CentO cores on the other nine chromosomes of Nipponbare are available, but both FISH analysis and genomic sequencing (Dong et al. 1998; Cheng et al. 2002) suggest that CentO clusters consisting of satellite repeat arrays physically coupled with the CRR sequences are a common feature of rice centromeres.

Active Genes As an unexpected result, dozens of putative genes, including active ones, have been identified in the recombination-suppressed centromere of rice Chr8 to encode protein sequences with the predicted functions, although their density is lower than that in the regions outside the centromeres (Nagaki et al. 2004; Wu et al. 2004). Seven of the 12 expressed genes, including four genes located within the functional domain region of the Chr8 centromere, moreover, are thought to be unique in the rice genome through Southern blot analysis. Sequencing and expression analysis of the centromeres of rice Chr3 (Yan et al. 2006), Chr4 (Zhang et al. 2004), and Chr5 (Mizuno et al. 2011) also identified active genes within the flanking regions of the CentO clusters, residing several kilobases from the CentO arrays. No genes seem to exist, however, within the CentO cluster regions.

3.2.3 Functional Domains of Centromere

Functional domains of centromeres can be usually identified through the analysis of DNA association with the centromere-specific histone CENH3, which is essential for chromosome segregation in all organisms. Within the genetically mapped centromere of Chr8, a region of ~750 kb, including a large cluster of three CentO tracts and 21 CRR elements, has been characterized as the kinetochore, a site for the attachment of chromosomes to spindle microtubules during mitosis and meiosis because the sequences of above CentO and CRR repeats bind rice CENH3 molecules (Nagaki et al. 2004). In the centromere of Chr3, the CENH3-binding region is large (~1.9 Mb) and includes ~440 kb of CentO repeats (Yan et al. 2006). A combination of chromatin immunoprecipitation and next generation sequencing suggests that the length of CENH3-binding domains on 9 of the 12 rice centromeres ranges from 0.4 to 1.2 Mb (Yan et al. 2008). Interestingly, the small cluster of CentO arrays (two short tracts) located proximal to the long arm of Chr8 does not co-localize with CENH3 (Nagaki et al. 2004), indicating that not all CentO repeats must be required for kinetochore formation on this chromosome (Fig. 3.3).

3.2.4 Centromere Evolution

The function of centromeres (to mediate faithful segregation of chromosomes in mitosis and meiosis probably through the interactions between CENH3 and centromeric DNA sequences of both satellite repeats and retrotransposons) is conserved, but their components and organization pattern involved in kinetochore formation vary remarkably, even between closely related species (Lamb and Birchler 2003; Ma et al. 2007b). Rice centromeres contain the 155-bp satellite repeats organized in tandem arrays that range from 60 kb to 1.9 Mb on different chromosomes. Centromeres in *Arabidopsis* (*Arabidopsis thaliana*), on the other hand, carry the tandem arrays of 180-bp satellite repeats, ranging from 0.6 to 1.8 Mb on its different chromosomes (Copenhaver et al. 1999). No sequence similarity of centromere-specific satellite repeats could be identified between the rice and *Arabidopsis*, and the similarity of satellite sequences between rice CentO and maize (*Zea mays*) CentC is restricted to short motifs (Cheng et al. 2002; Heslop-Harrison et al. 1999). The content and structure of rice centromeres are evolutionarily dynamic. The whole genome of the *japonica* rice cultivar Nipponbare, for example, contains a total of ~7 Mb of CentO satellite repeat sequences, about 2 Mb more than that of the *indica* rice cultivar Zhongxian 3037 (Cheng et al. 2002); their genomes diverged from that of the wild progenitor *O. rufipogon* about 0.44 million years ago (Khush 1997; Ma and Bennetzen 2004). Comparison of the orthologous centromeres on Chr8 between the two subspecies of *O. sativa* confirms the rapid diversification of centromere structure (Wu et al. 2009): the *indica* cultivar Kasalath has a large CentO core (~148 kb), about twice that of Nipponbare, because of the notable expansion of both CentO and CRR sequences (Fig. 3.3). The CentO satellite repeat sequence itself appears to be conserved within most of the *Oryza* species carrying the different types of genomes (AA–EE), although substitutions, insertions, and deletions have been detected (Dong et al. 1998; Zhang et al. 2005). However, another wild relative of *O. sativa*, *O. brachyantha*, with the FF genome, is likely to contain a different class of centromere-specific satellite repeat with no homology to CentO (Yi et al. 2013). Unlike the CentO repeat sequences, which are unique to rice, centromere-specific retrotransposons seem to be relatively conserved among grass species, suggesting positive selection for this type of LTR retrotransposons for the maintenance of centromere function (Jiang et al. 1996; Langdon et al. 2000). Structural and evolutionary dynamics of the centromere-specific retrotransposons are observed not only among different species but also between homologous chromosomes within the same species (Cheng et al. 2002; Nagaki et al. 2003). Within the genetically mapped Chr8 centromere region, about 45% of the LTR retrotransposon elements in Kasalath do not have orthologs in Nipponbare, indicating frequent and extensive DNA rearrangements caused by frequent unequal homologous recombination (Wu et al. 2009). In particular, many more CRR elements are accumulated within the CentO cluster of Kasalath than within that of Nipponbare; most of these elements appeared after the divergence of *indica* and *japonica* rice (Fig. 3.3). All

putative genes annotated within the Chr8 centromere region, on the other hand, are completely conserved between the two different rice cultivars of Kasalath and Nipponbare. Moreover, seven genes (five of them belonging to gene families assigned to physiological and/or cellular functions) within the functional domain of the Chr8 centromere are conserved even across the three different rice species (*O. sativa*, *O. glaberrima*, and *O. brachyantha*) (Fan et al. 2011). The two cultivated rice species, *O. sativa* and *O. glaberrima*, share the same type of genome (AA), which diverged from that of *O. brachyantha* (FF) 10–15 million years ago, strongly indicating that these genes are biologically essential, surviving even within a recombination-suppressed genomic region over a long evolutionary time. In summary, the existence of comparatively small CentO arrays and a number of active genes, as well as an elevated methylation level (Yan et al. 2010) in the CENH3-binding domain, suggest that the current centromeres of Nipponbare Chr8 (and probably also Chr4 and Chr5) might represent a stage of evolution intermediate between the genetic regions similar to human neocentromeres and fully mature centromeres composed of megabase-sized satellite repeats (Ma et al. 2007a, b; Nagaki et al. 2004).

3.3 Rice Telomere

In most organisms, telomeres located at each chromosome end contain DNA repeat arrays; they protect chromosome ends from deterioration or fusion with neighboring chromosomes. Because canonical telomere-specific repeat arrays do not contain enzyme cleavage sites, PAC or BAC libraries do not contain clones derived from these regions, and a fosmid library containing random mechanically sheared DNA has been used for telomere sequencing (Ammiraju et al. 2005; Mizuno et al. 2006).

3.3.1 Composition and Organization of Telomere Sequences

The ends of rice chromosomes are composed of satellite repeat sequences (Wu and Tanksley 1993; Yang et al. 2005) with the same consensus sequence (TTTAGGG) as in *Arabidopsis* (Richards and Ausubel 1988) and tomato (*Lycopersicon esculentum*) (Ganal et al. 1991). In the Nipponbare rice genome, the length of seven telomeres (measured by fiber-FISH) ranges from 5.1 to 10.8 kb, corresponding to 730–1500 copies of the TTTAGGG sequence (Mizuno et al. 2006). A total of 892 TTTAGGG copies, organized in tandem arrays, are assembled in the current Nipponbare genome sequence (IRGSP-1.0), partially covering the telomere regions on 14 of its 24 chromosomal ends (Fig. 3.2; Table 3.2).

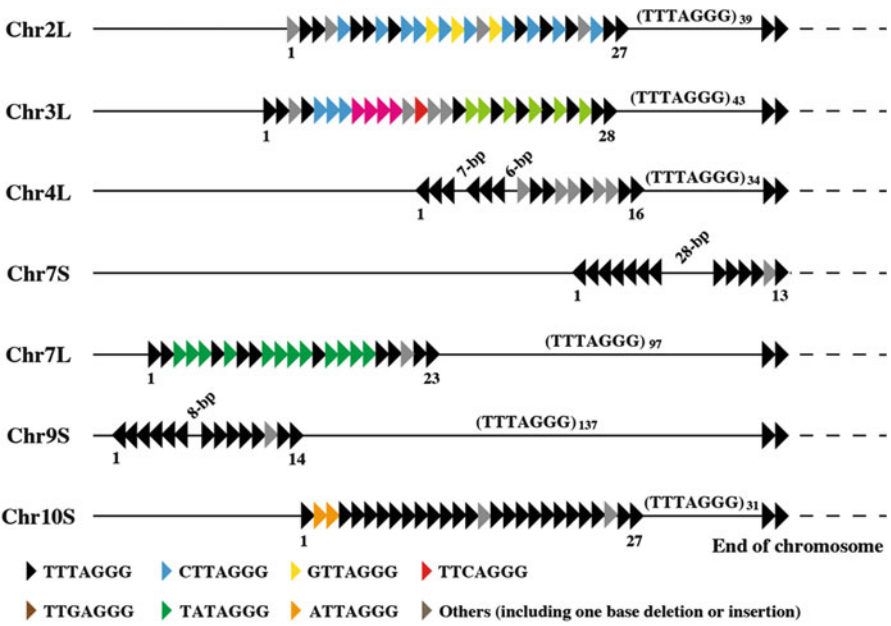

Fig. 3.4 Organization of telomere satellite repeats in rice. Numbers under satellite repeat copies represent their position (with arrowheads indicating their directions) at the junction between the chromosome-specific region and the telomere array. $(TTTAGGG)_n$ indicates the satellite repeats (n=copy number) residing towards the distal end, where a few copies containing a one-base (T) insertion (TTTTAGGG) or deletion (TTAGGG) are present

3.3.2 Sequence Variants of Telomere Satellite Repeat

Insertions of DNA sequences into satellite arrays and inverted satellite repeat sequences are located adjacent to the beginning of the telomere arrays on the ends of rice Chr4L, Chr7S, and Chr9S (Fig. 3.4). In Nipponbare, about 4.9% of all copies of the telomere satellite repeat sequenced so far show a one-base deletion (TTTAGGG->TTAGGG), suggesting preferential mutations of thymine (Mizuno et al. 2008a). At least six distinct types of the substituted variants of telomere satellite sequence (ATTAGGG, CTTAGGG, GTTAGGG, TATAGGG, TTCAGGG, and TTGAGGG) are arranged in mosaic blocks within the proximal telomere repeat arrays, indicating the sequence and structural divergence of telomere satellite repeats (Fig. 3.4). These mosaic blocks of noncanonical telomere sequences may have arisen from a rapid expansion of a single mutation rather than from gradual accumulation of random mutations because of the rapid rearrangement and frequent recombination (Li and Lustig 1996; Watson and Shippen 2007). The frequent accumulation of the above sequence variants (substitutions and deletions), particularly near the junction with the chromosome-specific region, likely reflects the nature of the telomere, in which the satellite

repeats are reconstructed (on an evolutionary time scale) in the distal region but rarely in the proximal region by telomerase (Sykorova et al. 2003).

3.3.3 Variation of Telomere Length

The length of telomere satellite repeat arrays varies among *Oryza* species (AA–HHJJ genomes) from 5 to 20 kb (Mizuno et al. 2006). It varies even within Asian cultivated rice (*O. sativa*), where the molecular weight is much lower in Nipponbare (*japonica*) than in Kasalath (*indica*). Considerable differences in telomere length (1.8–40.0 kb) have also been detected among maize inbred lines (Burr et al. 1992). The length of *Arabidopsis* telomeres is ~2.5 kb (Kotani et al. 1999) and is much smaller than that in tobacco (*Nicotiana tabacum*), where it reaches 160 kb (Fajkus et al. 1995a). These species- or chromosome-dependent variations might be a consequence of genetic or epigenetic differences in the subtelomere sequences that control the balance between telomere shortening and elongation (Majerova et al. 2014; Vaquero-Sedas and Vega-Palas 2014). In barley (*Hordeum vulgare*), telomere length decreases during differentiation but increases in callus culture (Kilian et al. 1995), whereas it is stable in *Melandrium album* during plant growth and development (Riha et al. 1998). In rice, a telomeric protein (RTBP1) binds to the duplex array of TTTAGGG repeats at chromosome ends (Ko et al. 2009). Future studies aimed at understanding the biological mechanisms that regulate the length of plant telomeres can be expected because telomere dynamics seem to link the lifestyle and lifespan in diverse taxa (Monaghan and Haussmann 2006).

3.3.4 Subtelomere

The subtelomere forms the junction between the chromosome-specific DNA sequence and the tandem array of telomere satellite repeats. Species-specific subtelomeric repeats have been reported in various plants such as barley (Kilian and Kleinhofs 1992), tobacco (Fajkus et al. 1995b), tomato (Ganal et al. 1991), and wheat (*Triticum aestivum*) (Mao et al. 1997). The physical locations of these repeats are closely associated with the telomere regions at almost all chromosome ends in barley (Roder et al. 1993) and tomato (Zhong et al. 1998). In rice, a repetitive subtelomeric sequence, TrsA (AA genome-specific tandem repeat sequence of 355 bp), exists in tandem arrays within genomic regions distal to chromosome ends; both the copy number and chromosomal location of this sequence vary among *Oryza* species (Ohtsubo and Ohtsubo 1994) and even cultivars (Ohtsubo et al. 1991). In Nipponbare, at least five chromosome arms (Chr5L, 6S, 8L, 9L, and 12L) carry TrsA arrays, which are arranged in discrete clusters of 2–106 copies in a

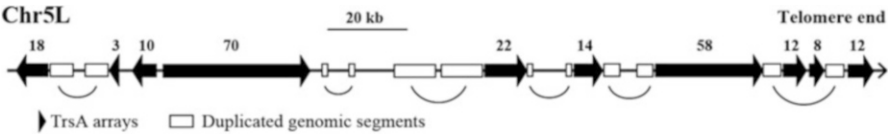

Fig. 3.5 Structure of the subtelomere on the long arm of rice chromosome 5. Numbers indicate the copy number of the TrsA sequence within each array, with the array direction shown by an arrow. Curves show segmental duplications within the TrsA cluster region

chromosome-specific manner rather than being distributed uniformly throughout subtelomeric regions (Mizuno et al. 2008b). These TrsA repeats reside very near (<1 kb) to telomere ends, except in Chr12L, where they are located about 200 kb from telomere satellite arrays. Intrachromosomal segmental duplications are likely involved in the accumulation and organization of TrsA repeats in the subtelomeric regions of Chr5L (Fig. 3.5) and Chr9L. Following the duplication, amplification of the TrsA repeat could also increase its copy number. Regions carrying TrsA repeat clusters do not contain expressed genes. These observations suggest that the subtelomeric regions on several rice chromosomes have an unusual structure and evolutionary history due to the integration and/or duplication of TrsA repeats, which may buffer the spread of gene silencing through the telomere position effect (Baur et al. 2001). In Nipponbare, at least 14 chromosome ends contain no TrsA repeats, indicating that telomere satellite arrays on these chromosome ends are directly attached to the gene-containing regions (Mizuno et al. 2008b). Interestingly, the telomere repeats on Chr9S abut the rDNA of the nucleolar organizer region (Fujisawa et al. 2006), similar to chromosomes 2 and 4 of *Arabidopsis* (The Arabidopsis Genome Initiative 2000).

3.4 Conclusions

Sequencing of rice centromeres and telomeres has revealed the compositional, structural, and evolutionary dynamics of these highly heterochromatic regions, which are important for chromosome transmission and genomic stability. This information furthers our understanding of what the centromere and telomere are and how they function in plants; it may also facilitate the development of advanced biological techniques for the production of artificial chromosome platforms in the near future.

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Chapter 4
Rice Organelle Genomics: Approaches to Genetic Engineering and Breeding

Tomohiko Kazama, Asuka Nishimura, and Shin-ichi Arimura

Abstract Although organelle (mitochondria and plastid) genomes have less than 1% of the genes in the nucleus, they encode essential genes, such as those involved in energy production, respiration, and photosynthesis, and genes that control agronomically important characteristics such as cytoplasmic male sterility. Organelle genomes have high copy numbers in each cell (one to two orders of magnitude greater than in the nucleus) and are characterized by maternal inheritance. To know functions of genes encoded in the organelle genomes or to develop new plants adapted to various severe environments, genetic engineering of organelle genomes is one of the promising approaches. However, modifying the mitochondrial or plastid genomes in rice is presently impossible or difficult. Here, we discuss the characteristic features of these genomes and recent attempts at plastid transformation.

Keywords Mitochondria · Plastid · Chloroplast · Organelle genome

4.1 Mitochondrial Genomics in Rice

The common function of mitochondria in plants and animals is to act as an energy center to create ATP by oxidative phosphorylation. Despite this similarity, the genome sizes and structures of the mitochondrial genome in plants are quite different from those in animals. The size of the mitochondrial genome ranges

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from 200 to 11,300 kb in plants, while it is only about 17 kb in mammals, apparently as a result of the loss of many fragments. The large size of plant mitochondrial genome is mostly due to the presences of repeated sequences, noncoding regions, and introns rather than the increased number of genes.

4.1.1 Rice Mitochondrial Genome Sequencing

In flowering plants, the mitochondrial genome was first sequenced in *Arabidopsis thaliana* (Unseld et al. 1997) and subsequnetly in the rice *japonica* cultivar Nipponbare (Notsu et al. 2002). The genomes were sequenced by preparing phage clone libraries and restriction maps, followed by sequencing each clone. The rice mitochondrial genome was initially proposed to consist of five basic circular DNAs, with each circle sharing one or two fragments identical to those in other circles (Iwahashi et al. 1992). A master circle was hypothesized to form by homologous recombination between those identical fragments in the five circular DNAs (Iwahashi et al. 1992; Notsu et al. 2002).

So far, the mitochondrial genomes of 14 rice strains including wild rice have been reported (Table 4.1). The sizes of the genomes range from 402 kb (WA-type cytoplasmic male sterility, WA-type CMS) to 638 kb (IR 6888B, a maintainer line of WA-type CMS). The G + C contents of each genome are very similar. Except BT-type CMS, the genomes are hypothesized to have a single master-circle structure. Although the BT-type CMS mitochondrial genome is possible to have the same structure, a Southern blot analysis suggests that it consists of two separate circular molecules (Kazama and Toriyama 2016).

4.1.2 Gene Contents and Genes Associated with Cytoplasmic Male Sterility

The Nipponbare mitochondrial genome is reported to contain 35 genes for known proteins, 3 ribosomal RNAs, 2 pseudo-ribosomal protein genes, 17 tRNAs, and 5 pseudo-tRNAs (Notsu et al. 2002). Some genes consist of multiple exons that are dispersed throughout the mitochondrial genome and that are trans-spliced to form functional mRNAs (Table 4.2). Although 19 open reading frames (ORFs), which encode over 150 amino acids, have also been predicted, transcriptional products were detected in only 10 of those ORFs (Notsu et al. 2002). The existence of all genes except the predicted ORFs was confirmed in reported rice mitochondrial genome sequences (Tian et al. 2006; Fujii et al. 2010; Bentolila and Stefanov 2012; Zhang et al. 2012; Igarashi et al. 2013; Okazaki et al. 2013; Asaf et al. 2016;

Table 4.1 A summary of reported rice mitochondrial genome sequence

Accession no.	Strain	Organism	Length (bp)	% GC	References
BA000029	Nipponbare	*Oryza sativa japonica* group	490,520	43.9	Notsu et al. (2002)
DQ167399	93-11	*Oryza sativa indica* group	491,515	43.8	Tian et al. (2006)
DQ167400	Nipponbare S	*Oryza sativa japonica* group	490,669	43.8	Tian et al. (2006)
DQ167807	PA64S	*Oryza sativa japonica* group	490,673	43.8	Tian et al. (2006)
AP011076	CW-CMS	*Oryza rufipogon*	559,045	44.0	Fujii et al. (2010)
AP011077	LD-CMS	*Oryza sativa indica* group	434,735	43.9	Fujii et al. (2010)
JF281153	IR 6888B	*Oryza sativa indica* group	637,692	43.9	Bentolila and Stefanov (2012)
JF281154	WA-CMS	*Oryza sativa indica* group	401,567	43.9	Bentolila and Stefanov (2012)
JN861111	Hassawi	*Oryza sativa indica* group	454,820	43.8	Zhang et al. (2012)
JN861112	IR 1112	*Oryza sativa indica* group	454,894	43.8	Zhang et al. (2012)
AP012527	RT98-CMS	*Oryza rufipogon*	525,913	44.2	Igarashi et al. (2013)
AP012528	RT102-CMS	*Oryza rufipogon*	502,250	44.0	Okazaki et al. (2013)
KU176938	W1340	*Oryza minuta*	515,022	44.0	Asaf et al. (2016)
AP017385	BT-CMS	*Oryza sativa indica* group	95,643[a]	44.0	Kazama and Toriyama (2016)
AP017386	BT-CMS	*Oryza sativa indica* group	440,134[b]	43.9	Kazama and Toriyama (2016)

[a]Subgenome 1
[b]Subgenome 2

Kazama and Toriyama 2016). The sequence complexity of plant mitochondrial genomes sometimes makes new sequences and ORFs via illegitimate homologous recombination. Expression of such new ORFs sometimes leads to male sterility, called cytoplasmic male sterility (CMS). Several studies have identified CMS-associated genes (or CMS-causative genes) (Iwabuchi et al. 1993; Akagi et al. 1994; Fujii et al. 2010; Bentolila and Stefanov 2012; Okazaki et al. 2013; Igarashi et al. 2013). Previously, CMS-associated genes have been identified by comparisons of gene structures and their expression profiles between CMS-causing and normal mitochondria. In rice, CMS lines are bred by cytoplasmic substitution via repeated backcrossing. In this case, the cytoplasmic donor cultivar carries *restorer of fertility* (*Rf*) genes in its nuclear genome. This indicates that the

Table 4.2 Location of homologous fragments of the mitochondrial genes in the nuclear genome

Gene	Function	Location in the nuclear genome				
		Exon1	Exon2	Exon3	Exon4	Exon5
nad1	Complex I	N.D.	chr. 1	chr. 8	chr. 9	chr. 12
nad2	Complex I	chr. 9	chr. 9	chr. 1	chr. 1	chr. 1
nad3	Complex I	chr. 12	–			
nad4	Complex I	chr. 12	chr. 12	chr. 12	chr. 12	–
nad4L	Complex I	N.D.	–			
nad5	Complex I	chr. 12	chr. 12	chr. 12	N.D.	N.D.
nad6	Complex I	chr. 12	–			
nad7	Complex I	chr. 12	chr. 12	chr. 12	chr. 12	chr. 12
nad9	Complex I	chr. 1	–			
cob	Complex III	N.D.	–			
cox 1	Complex IV	chr. 12	–			
cox 2	Complex IV	N.D.	chr. 12	–		
cox 3	Complex IV	N.D.	–			
atp1	Complex V	chr. 9	–			
atp4	Complex V	chr. 12	–			
atp6	Complex V	chr. 1	–			
atp8	Complex V	chr. 12	–			
atp9	Complex V	chr. 12	–			
ccmB	Cytochrome c	chr. 12	–			
ccmC	Cytochrome c	chr. 12	–			
ccmFc	Cytochrome c	chr. 12	N.D.	–		
ccmFn	Cytochrome c	N.D.	–			
mat-r	Transcription	N.D.	–			
rps1	Translation	N.D.	–			
rps2	Translation	chr. 12	–			
rps3	Translation	chr. 12	chr. 12	–		
rps4	Translation	N.D.	–			
rps7	Translation	chr. 12	–			
rps11	Translation	N.D.	–			
rps12	Translation	chr. 12	–			
rps13	Translation	chr. 12	–			
rps14	Translation	chr. 9	–			
rps19	Translation	N.D.	–			
rpl2	Translation	N.D.	N.D.	–		
rpl5	Translation	chr. 9	–			
rpl16	Translation	chr. 12	–			
orfX	Transporter	chr. 12	–			

Sequences existed in nuclear genome entirely are listed
N.D. means full-length sequences are not detected in the nuclear genome

cytoplasmic donor cultivar has both CMS-associated and *Rf* genes; RF suppresses the expression of the CMS-associated gene, and male sterility does not occur. The most common approach to identify CMS-associated genes has mostly relied on Northern blot screening between CMS and fertility restored lines (Hanson and Bentolila 2004).

Recently, it has become more common to use next-generation sequencing to identify CMS-associated genes. Whole mitochondrial genomic sequences of CMS rice were obtained and compared between those of standard cultivars, such as Nipponbare, to screen for new ORFs that are absent from the reference genome (Bentolila and Stefanov 2012; Kazama and Toriyama 2016). Then, CMS-associated gene candidates were selected based on the criteria that they are chimeric in structure with known mitochondrial genes or encode peptide containing a transmembrane domain, because all reported CMS-associated genes have such characteristics. Subsequently, the expressions of candidate genes were checked to determine whether they exhibit different patterns in the presence or absence of *Rf* genes. Expression pattern differences that depend on the presence or absence of *Rf* genes indicate that the expression of CMS-associated genes is the cause of CMS. However, it is presently impossible to modify a plant mitochondrial genome by DNA fragment knock-in and specific ORF knock-out. Thus, there is not yet an absolute proof that a CMS-associated gene is a CMS-causative gene that has not been obtained.

4.1.3 Mitochondrial DNA Fragments in the Nuclear Genome

Many DNA fragments identical to mitochondrial DNA have been found in the nuclear genome (Notsu et al. 2002; Bentolila and Stefanov 2012). This kind of nuclear fragment is called "promiscuous" DNA and thought to be translocated from mitochondria through evolutionary processes and/or organelle establishment. In *Arabidopsis*, a 620-kb insertion of a mitochondrial genome sequence was reported on chromosome 2 (Stupar et al. 2001). In rice, fragments representing 60% of the mitochondrial genome were found in the nuclear genome (Bentolila and Stefanov 2012). Interestingly, such promiscuous DNA fragments are unequally distributed among the chromosomes.

To identify which mitochondrial genes exist in the nuclear genome, BLAST search was performed (Table 4.2). In this analysis, genes except tRNAs and the predicted ORFs in the Nipponbare mitochondrial genome were used as queries. Homologous sequences were found for all but 14 mitochondrial genes (Table 4.2) (International Rice Genome Sequencing Project 2005). The similarity between the mitochondrial and nuclear gene sequences was more than 88%. This indicates that almost all of the mitochondrial genes in rice have been translocated into the nuclear genome and that the translocation events of mitochondrial genome could have occurred in relatively recent times.

4.2 Chloroplast Genomics in Rice

4.2.1 Use of Rice Chloroplast Genomes for Comparative Genomics

Chloroplasts are organelles that are representative of plants; they are the most prominent plastid present in green tissues, and play a crucial role in plants as a metabolic center involving various biochemical processes, including photosynthesis. Chloroplasts possess their own genome, which is generally circular and consists of a large single copy (LSC) region and a small single copy (SSC) region, respectively, that separate two inverted repeat regions (IRs). The chloroplast genome contains three major groups of genes that encode (i) subunits of the photosynthetic complex, (ii) components of the gene expression machinery, and (iii) a few proteins that may function in metabolic pathways and unknown proteins (Shimada and Sugiura 1991). Compared with the mitochondrial genome, the chloroplast genome of land plants has a well-conserved structure, although the gene content, gene order, and fine structural details depend on the species (Jansen et al. 2007; Xu et al. 2015). In the grass family (Poaceae), the chloroplast genome shares some structural arrangements that are different from those of other plant families (Doyle et al. 1992; Katayama and Ogihara 1996; Saski et al. 2007). These include three inversions in the LSC; deletions of *accD*, *ycf1*, and *ycf2*; and loss of introns in *clpP* and *rpoC1* (Katayama and Ogihara 1996; Saski et al. 2007; Daniell et al. 2016).

The rice chloroplast genome was first sequenced in the *Oryza sativa* L. cv. Nipponbare (Hiratsuka et al. 1989); its length was 134,525 bp with 38.99% GC content. The present annotation data (https://www.ncbi.nlm.nih.gov/nuccore/NC_001320.1) show that the Nipponbare chloroplast genome contains 159 genes, including 108 protein-coding genes, 8 ribosomal RNA genes, 38 transfer RNA genes, and 5 pseudogenes. To date, more than 100 rice chloroplast (plastid) genome sequences, including that of wild rice, are available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide/) because of advances in sequencing technologies.

Oryza, which includes 23 species, is divided into 10 nuclear genome types, which include 6 diploids (AA, BB, CC, EE, EF, and GG) and 4 allotetraploids (BBCC, CCDD, HHJJ, and HHKK) (Ge et al. 1999). The AA genome type includes three cultivated rice species (*Oryza sativa* ssp. *japonica* and *O. sativa* ssp. *indica* in Asia and *O. glaberrima* in Africa) and six wild relatives: *O. barthii*, *O. glumaepatula*, *O. longistaminata*, *O. meridionalis*, *O. nivara*, and *O. rufipogon*. The Asian cultivated rice, *O. sativa*, is generally thought to have originated from *O. rufipogon* (Khush 1997; Vaughan et al. 2008; Huang et al. 2012). However, despite numerous studies (Ma and Bennetzen 2004; Vitte et al. 2004; Zhu and Ge 2005; Londo et al. 2006; Molina et al. 2011; Huang et al. 2012; Yang et al. 2012; Civáň et al. 2015; Huang and Han 2015), the domestication history remains under debate.

Recent studies of the evolutionary and phylogenetic relationships among the AA genome species based on whole chloroplast genome sequences showed that two subspecies of *O. sativa*, *japonica* and *indica*, had distinct maternal origins (Wambugu et al. 2015; Kim et al. 2015; Tong et al. 2016). It is indicated that the maternal genome of *japonica* has been derived from *O. rufipogon*, whereas the *indica* maternal genome has been originated from *O. nivara*, although the nuclear genomes seem to be of complex origin (Kim et al. 2015). Nuclear genome analyses suggest that the African cultivated rice *O. glaberrima* was domesticated from *O. barthii* (Li et al. 2011b; Wang et al. 2014). Phylogenetic relationships of several chloroplast genomes also indicated that the maternal origin of *O. glaberrima* was *O. barthii* (Wambugu et al. 2015; Kim et al. 2015; Tong et al. 2016). Moreover, chloroplast genome analyses of worldwide rice accessions revealed that South American wild rice, *O. glumaepatula*, has a distinct chloroplast genome (Kim et al. 2015), and *O. rufipogon* contains cytoplasm derived from different geographic regions (Kim et al. 2015; Wambugu et al. 2015). Chloroplast-based phylogenies usually reflect geographic distributions better than nuclear genomes and should be useful for resolving the evolutionary and domestication history of rice. Wild species have disease-resistance and stress-tolerance genes, which make them valuable genetic resources for rice improvement and breeding (Brar and Khush 1997). In recent years, wild species with the EE genome (*O. australiensis*) and BBCC genome (*O. minuta*) chloroplast sequences were also reported (Nock et al. 2011; Waters et al. 2012; Asaf et al. 2017). These chloroplast genome studies will elucidate the detailed genetic relationships among rice species and will be helpful in developing future breeding strategies using new genetic resources. In addition, the accumulated rice chloroplast sequences also provide insight for chloroplast genome engineering to improve chloroplast function such as photosynthetic capacity.

4.2.2 Perspectives on Rice Chloroplast Genome Engineering

In higher plants, the first successful chloroplast transformation was achieved in tobacco by particle bombardment about 30 years ago (Svab et al. 1990). Chloroplast transformation has many advantages over nuclear transformation, including a high level of transgene expression, multigene transformation, site-directed gene integration, absence of gene silencing, and maternal inheritance that prevents transgene flow into the environment. Each plant cell has dozens of chloroplasts, each of which contains multiple genome copies. Thus, gene expression can be ten to a hundred times greater in chloroplast genomes than in nuclear genomes. With this property, plants can be used to make large amounts of foreign proteins, such as pharmaceutical proteins (e.g., vaccine antigens and antibiotics) and industrial enzymes (e.g., hydrolases, redox enzymes, and transferases). Many functional proteins have been expressed in tobacco and lettuce chloroplasts (reviewed in Jin and Daniell 2015;

Daniell et al. 2016), and expression can be as high as 70% of total leaf protein (Ruhlman et al. 2010). Chloroplasts have great potential as bioreactors for commercial production. Now, many therapeutic proteins are in clinical development (Zhang et al. 2017).

Chloroplast transformation has also been used to control agronomic traits, including resistance to insects, diseases, and herbicides, and abiotic stress tolerance of plants (reviewed in Jin and Daniell 2015; Daniell et al. 2016). For example, double-stranded (ds) RNA expressed in chloroplasts was shown to induce RNA interference (RNAi) of target genes in insect hosts (Jin et al. 2015; Zhang et al. 2015), demonstrating a new approach to protecting crops without chemicals.

Chloroplast engineering of metabolic pathways or photosynthetic ability could be used for crop improvement (Maliga and Bock 2011; Hanson et al. 2013; Wani et al. 2015). To manipulate the isoprenoid pathway, seven genes were simultaneously inserted into the chloroplast genome, resulting in the accumulation of high levels of target metabolites (Kumar et al. 2012). Photosynthetic efficiency was also enhanced by replacing the tobacco chloroplast *rbcL* gene with three genes from the cyanobacterium *Synechococcus* (*rbcL*, *rbcS*, and an assembly gene) (Lin et al. 2014).

Despite the remarkable progress in chloroplast transformation technologies, there are still substantial limitations to their use. Successful examples of chloroplast genome engineering have been restricted to only tobacco and a few dicots (Jin and Daniell 2015). Development of fully chloroplast-engineered monocots has not yet been achieved because of the lack of transformation protocols (Bock 2007; Clarke and Daniell 2011; Khan 2012; Rigano et al. 2012). Major obstacles to chloroplast transformation in monocots are thought to be (i) difficulty of tissue culture and regeneration from green tissue and (ii) no effective selection systems for retaining the transformed chloroplasts (plastids) and facilitating the transition from heteroplasmic to homoplasmic state (that is, all chloroplast genomes are replaced with identical transformed chloroplast genomes). Indeed, efficient protocols for tobacco chloroplast transformation were established based on tissue culture systems of green leaf materials using spectinomycin selection with the aminoglycoside 3″-adenyltransferase gene, *aadA*, as a selection maker (Lutz et al. 2007; Verma and Daniell 2007; Scotti and Cardi 2012). Unfortunately, most monocotyledonous plants are endogenously resistant to spectinomycin, because of the point mutations in their targeted *16S rRNA* gene (Fromm et al. 1987). Additionally, regenerable tissue culture materials in monocots are generally nongreen tissues, such as dark-grown embryogenic calli or suspension cells. In nongreen tissues, a small number of plastids exist as undifferentiated plastids called proplastids. Proplastids are about fivefold smaller in size than chloroplasts, and the gene expression levels are lower than those of chloroplasts (Vera and Sugiura 1995; Sakai et al. 1998; Silhavy and Maliga 1998; Daniell et al. 2002; Pyke 2007; Liebers et al. 2017).

Even with these disadvantages, several advancements have been reported in monocotyledonous cereal crops, particularly in rice. Khan and Maliga (1999) were the first to transform rice plastids. They used a fluorescent antibiotic resistance gene, *aadA11gfp*, a fusion gene of the *Aequorea victoria* green fluorescence protein

gene (*gfp*) and *aadA*. They introduced the fusion gene to rice suspension culture cells and selected the transplastomic lines with streptomycin: *aadA* confers resistance to both spectinomycin and streptomycin, and rice cells are sensitive to streptomycin. Twelve shoots regenerated from surviving cells on streptomycin-containing medium from 25 bombarded plates. The plastid transformation was proven by PCR detection of the transgene, but the GFP fluorescence was only observed in a few populations of highly heteroplasmic cells, including wild-type and transgenic chloroplasts. Despite this success, the authors did not indicate inheritance of the transgene or the fertility of the transplastomic plants. Subsequently, Lee et al. (2006) again attempted to introduce *gfp* and *aadA* by using streptomycin selection with the conventional rice cell culture with mature seed-derived calli. They successfully produced fertile transplastomic rice plants and demonstrated transmission of both transgenes to the progeny. However, the transformation efficiency was quite low, with two transgenic lines out of approximately 4000 calli on 100–120 bombarded plates; and all transplastomic lines still have been in heteroplasmic state.

A different selection approach using PPT (L-phosphinotricin), the active ingredient of the herbicide Basta, and the resistance gene *bar* was also attempted for rice plastid transformation (Li et al. 2009). Although the transformation efficiency was not clear, six transplastomic plants were obtained with various heteroplasmic levels, and maternal transmission of *bar* was suggested based on crossing experiments (Li et al. 2009). More recently, Li et al. (2016a, b) tried other selection strategy using a rice-specific chloroplast transformation vector with hygromycin-resistance cassette that contains the hygromycin phosphotransferase gene *hpt*. The antibiotic hygromycin B blocks protein biosynthesis in both prokaryotic and eukaryotic cells (Gonzalez et al. 1978), and hygromycin selection was widely used for nuclear transformation in rice (Hiei and Komari 2008). Fertile transplastomic plants were obtained, and transgene inheritance in progeny was demonstrated. However, only a few transformation events occurred in these experiments (24 surviving plants with approximately 20,000 total calli), and the number of transformed plastids was very small, probably because of weak selection pressure (Li et al. 2016b).

A possible reason for the lower transformation efficiencies observed in nontobacco species is reduced activity of plastid homologous recombination (Sikdar et al. 1998). Assuming this was the case, the application of transcription activator-like effector nucleases (TALENs) was tested in rice plastid transformation (Li et al. 2016a). TALENs are a powerful tool for targeted genome modification (Bogdanove and Voytas 2011). They induce genomic double-strand breaks (DSBs) for gene insertion and thus greatly stimulate the homologous recombination DNA-repair pathway (Wyman and Kanaar 2006). To improve the insertion efficiency of exogenous DNA fragments into the rice plastid genome by homologous recombination, Li et al. (2016a) conducted co-transformation of the TALEN vector and plastid transformation vector into rice calli by particle bombardment. As a result of preliminarily PCR analyses of T_0 plants, the transformation efficiency

(detection frequency of transgene fragments) of co-transformation (0.35%) was much higher than that of transformation with a single vector (0.11%).

In summary, despite the interest in chloroplast transformation of rice, it has not yet been successful. Issues to be solved include (i) choice of target tissues for gene introduction, (ii) activation of homologous recombination, and (iii) development of efficient selection methods. The candidate target tissues (plastids) include nongreen tissue (proplastids) and green tissue (chloroplasts). As mentioned above, calli, a nongreen tissue used in conventional tissue culture systems, contain small proplastids with low introduction efficiency and low levels of gene expression. In contrast, green tissue contains large chloroplasts that are thought to have high efficiency and high levels of gene expression. However, no culture system capable of regenerating green tissues has yet been established in rice. In maize, a leaf-based culture system has been constructed (Ahmadabadi et al. 2007). We attempted a similar culture method using rice seedlings, and confirmed that it can be used to culture (unpublished data). We are now testing this green tissue to see if it can be used for chloroplast transformation of rice.

The selection methods, for transplastomic rice plastids, streptomycin-*aadA*, PPT-*bar*, and hygromycin-*hpt*, all failed to achieve homoplasmy, which indicates that these selection methods are not suitable for rice plastid transformation. In dicots, a few alternative selection systems have recently been developed for plastid transformation (Barone et al. 2009; Li et al. 2011a; Bellucci et al. 2015; Dunne et al. 2014; Yu et al. 2017). The selection methods for dicots and other novel selection methods need to be tested for transforming rice plastids.

Furthermore, because of their low plastid transformation efficiency, monocots, including rice, seem refractory to integration of foreign genes into the plastid genome. Perhaps monocots have specific biological mechanisms to prevent it. At present, many aspects of monocot plastid biology remain unclear. Elucidation of the molecular mechanisms of plastid genome maintenance will be indispensable for establishing methods for plastid transformation in rice.

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Chapter 5
Genetic and Epigenetic Regulation of Meiotic Fate Decision and Gametophyte Specification in Rice

Ken-Ichi Nonomura, Seijiro Ono, and Kenji Ueda

Abstract The life cycle of sexual organisms is achieved through repeated rounds of fertilization and meiosis. After premeiotic DNA replication, plant meiosis produces four haploid spores by two sequential cell divisions without DNA replication. In most model organisms, including rice, the homologous chromosome pair is separated to opposite poles during meiosis I, and sister chromatids are separated during meiosis II. In animals, meiotic products directly mature into gametes, namely, sperms and eggs. In contrast, meiosis of land plants produces spores that undergo further somatic cell division and eventually form a multicellular haploid body containing sperms or eggs. In other words, land plants have two distinct multicellular bodies, sporophytic diploid and gametophytic haploid bodies. This type of reproductive mode is called alternation of generations and is commonly found in all land plants, some algae and fungi (Graham, Am Sci 73:178–186, 1985). These facts imply that plants have evolved unique genetic systems for reproduction, in addition to systems common to non-plant species. In this chapter, we overview the genetic and epigenetic systems regulating meiosis and gametogenesis and introduce challenges to improve the efficiency of breeding methods in rice (*Oryza sativa* L.).

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5.1 Introduction

Meiosis is a special type of cell division that contributes to an increase in genetic diversity of offspring via meiotic recombination. It also surveys for incompatibilities in ploidy levels and chromosomal structures between both parents in F_1 hybrids. This surveillance acts as a meiotic barrier to genetic exchanges with other species and consequently to preserve specific gene sets adaptive for respective habitats. During postmeiosis, angiosperm species (flowering plants) produce pollen and embryo sacs, which are extremely simplified gametophytes compared to those of other land plants. Thus, despite their tiny structures, complicated genetic mechanisms regulate development of pollen and embryo sacs.

The central topic of this review is male reproductive events, because in rice, the overwhelming number of male cells produced facilitates collection of more samples than the single female cell per flower, and studies of male reproduction can suggest approaches for applied breeding methods, such as male sterility-assisted hybrid production (Wilson and Zhang 2009) and development of varieties tolerant to low- or high-temperature injury during pollen development (Endo et al. 2009; Suzuki et al. 2015). Cell-cell communication between somatic and germ cells is one of the important aspects of meiosis and gametogenesis and thus is also described in this chapter.

5.2 Sexual Organogenesis

Primordial germ cells, called archesporial cells in plants, are initiated at the L2 layer of four-lobed anther primordia (Raghavan 1988) and at L2 of ovule primordia (Yamaki et al. 2011) (Fig. 5.1a). In anther development, archesporial cells divide and differentiate to sporogenous cells along the central axis of each anther locule and to the primary parietal cell layer that wraps around the central sporogenous cells. Primary parietal cells are somatic meristemoids and divide periclinally to make the endothecium and secondary parietal cells, which further divide periclinally and differentiate into the middle layer and tapetum (Raghavan 1988; Goldberg et al. 1993) (Fig. 5.1a). Tapetum-surrounded microsporangium is a nursery for pollen mother cells (PMCs) and microspores, both of which are sporogenous-cell derivatives. In this chapter, archesporial cells, sporogenous

cells, and meiocytes are defined as the plant germline or germ cells, equivalent to postmeiotic microspores and sperm cells, because they are specified to produce gametes by many reproduction-specific genes, as described below.

Rice MULTIPLE SPOROCYTES1 (MSP1) is a leucine-rich repeat (LRR) receptor-like protein kinase that is expressed within parietal cell derivatives in anthers and within hypodermal nucellar cells in ovules and determines the identity of *MSP1*-expressing cells (Nonomura et al. 2003) (Fig. 5.1b). The loss of MSP1 results in malformation of undifferentiated anther walls and, instead, in an increase in the number of both male and female germ cells. This observation suggests that a repressive function of MSP1 leads a subset of archespore-derived cells to be somatic nursery cells, but not to be the germline, in anthers and ovules (Nonomura et al. 2003). Rice TAPETUM DETERMINANT1 (TPD1)-LIKE1A (TDL1A) is a small peptide acting as a ligand that potentially binds the LRR of MSP1 (Zhao et al. 2008). The rice MSP1-TDL1A system is conserved in *Arabidopsis* as the receptor kinase EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS (EXS) (Zhao et al. 2002; Canales et al. 2002) and the peptide ligand TPD1 (Yang et al. 2003). TPD1 is expressed in sporogenous cells, suggesting that parietal cell fate determination depends on the central male germline (Yang et al. 2005; Jia et al. 2008). In rice, *TDL1A* mRNA is expressed in *MSP1*-expressing cells (Zhao et al. 2008), different from the spatial expression pattern of orthologous *TPD1* and *EMS1* genes in *Arabidopsis*.

Various transcription factors (TFs), especially basic helix-loop-helix (bHLH)-type proteins, are central keys to tapetum differentiation and development. TAPETUM DEGENERATION RETARDATION (TDR)- INTERACTING PROTEIN2 (TIP2)/bHLH142 determines the cell fate of middle layers and tapetum (Fu et al. 2014; Ko et al. 2014) and activates transcription of *TDR/bHLH5*. TDR can dimerize with TIP2 to promote tapetum differentiation and activates transcription of *CYSTEINE PROTEASE1* (*CP1*) and *ANTHER-SPECIFIC PROTEIN6* (*C6*) expression (Li et al. 2006), which have roles in programmed cell death (PCD) of tapetum and pollen wall formation, respectively (Lee et al. 2004; Zhang et al. 2010). ETERNAL TAPETUM1 (EAT1)/bHLH141, a phylogenic paralog of TIP2 (Carretero-Paulet et al. 2010), also dimerizes with TDR and promotes transcription of tapetal PCD inducers *ASPARTYL PROTEASE25* (*AP25*), *AP37*, and *C6* (Niu et al. 2013b; Ji et al. 2013a). UNDEVELOPED TAPETUM1 (UDT1)/bHLH164 is also involved in regulatory cascades to develop anther walls properly (Jung et al. 2005). Besides bHLH TFs, rice GAMYB, an R2R3-type MYB TF, is required for gibberellic acid (GA) signaling in tapetal PCD and secretion of pollen coat materials (Kaneko et al. 2004, Tsuji et al. 2006). Indeed, GAMYB directly targets multiple genes involved in tapetal PCD and lipid biosynthesis (Aya et al. 2009). Rice PERSISTENT TAPETAL CELL1 (PTC1), a TF with a PHD-finger homeodomain, also promotes tapetal PCD and lipid metabolism genes (Li et al. 2011a).

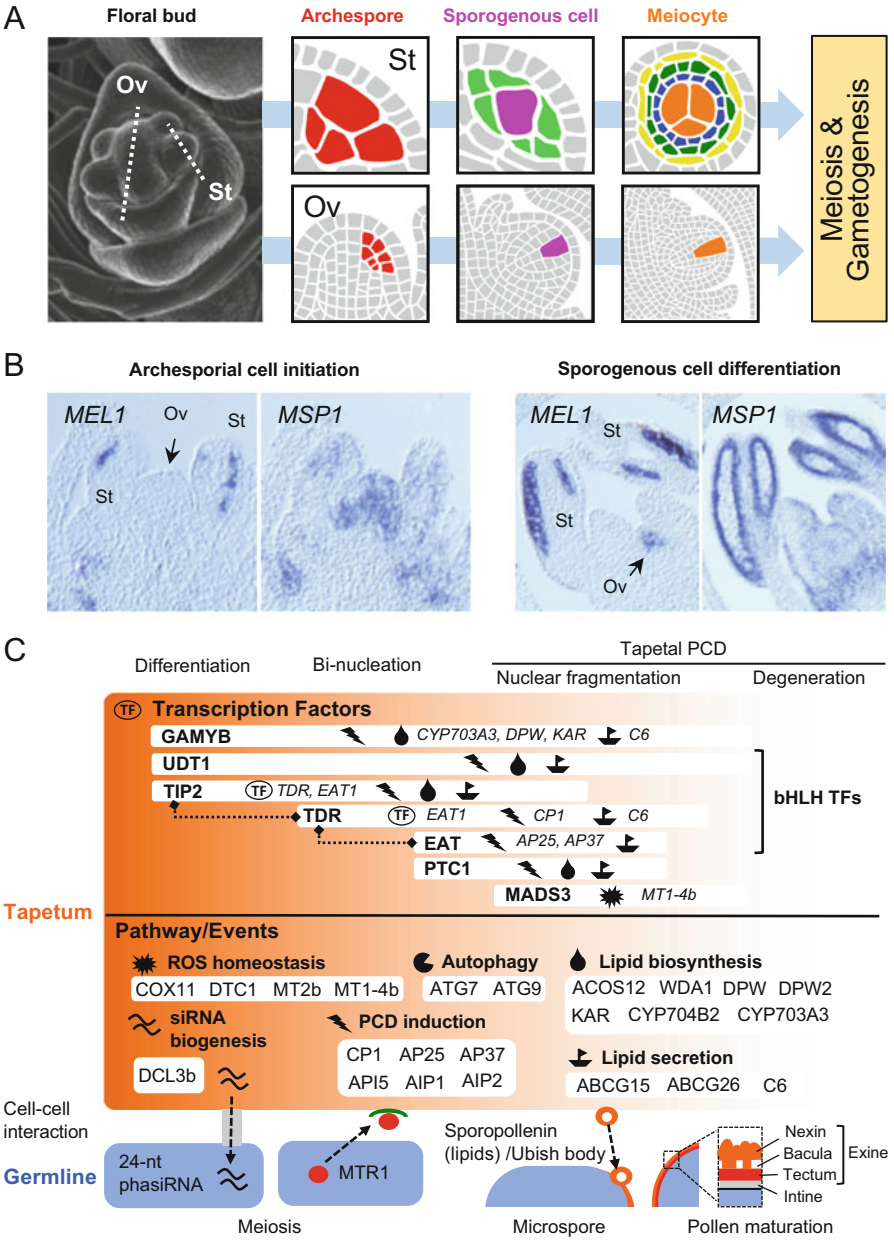

Fig. 5.1 Early reproductive organogenesis and gene expression in rice. (**a**) Schematics of cross sections of stamen (St) and longitudinal sections of ovule (Ov), sliced at dotted lines on the scanning electron microscopic image of a rice floral bud. The photo and illustrations are quoted from Nonomura et al. (2011) with slight modifications. Red cells; archesporial cells, magenta; sporogenous cells, light green; parietal cells, yellow; endothecium, dark green; middle layer, blue;

5.3 Germline Initiation and Meiotic Cell Fate Decision

MEIOSIS ARRESTED AT LEPTOTENE1 (MEL1) is a germline-specific Argonaute protein having essential roles in meiosis I in rice (Nonomura et al. 2007) (Fig. 5.1b). *MEL1* mRNA begins to accumulate in male and female archesporial initials, suggesting that the meiotic cell fate decision starts at the initial stage of germline development. The upstream signaling inducing archesporial cells is unclear. *MEL1* is expressed in L2 cells just beneath the epidermis transiently expressing *LONELY GUY* (Yamaki et al. 2011), which encodes an enzyme activating cytokinin to maintain the meristem activity (Kurakawa et al. 2007), implying the importance of phytohormone signaling in germline induction. In maize, hypoxia is essential to induce archesporial cells (Kelliher and Walbot 2012, 2014). In *Arabidopsis*, AGAMOUS, the MADS-box TF specifying stamens and carpels, directly activates transcription of SPOROCYTELESS/NOZZLE (Ito et al. 2004), which encodes a putative TF determining sporogenous cell fate (Yang et al. 1999; Schiefthaler et al. 1999).

Another major step to establish meiotic cell fate is the decision to switch from the mitotic cell cycle to the meiotic cycle (Kimble 2011). This meiotic entry event is genetically regulated. In plants, the first reported gene controlling the meiotic entry was maize *AMEIOTIC1* (*AM1*) (Palmer 1971; Golubovskaya et al. 1993), which is a plant-specific protein with a short-coiled coil motif conserved in *Arabidopsis* (*SWITCH1* (*SWI1*), Boateng et al. 2008; Mercier et al. 2001) and rice

Fig. 5.1 (continued) tapetum, *mandarin*; pollen mother cells (PMCs). Interestingly, multiple archesporial cells are initiated in Ov (Nonomura et al. 2007), whereas only one of them finally matures into single embryo-sac mother cell (female meiocyte). This suggests that archespore-derived somatic cells surrounding the germline also have potential to be the germline, which is suppressed by *MSP1* function (see (**b**)). (**b**) In situ expression of *MEL1* and *MSP1* mRNAs (dark blue regions) on longitudinal sections of developing flowers. Two adjacent images in each combination photo are obtained from serial sections of the same flower. *MEL1*, but not *MSP1*, begins to express in St (left) and subsequently in Ov (no photo) at the archespore initiation stage. Subsequently, archespores produce *MEL1*-expressing sporogenous cells and *MSP1*-expressing parietal cells, which are never overlapped with each other (right). (**c**) Genetic regulation of tapetum development and cell-cell communication with the male germline. Both cell-autonomous and non-cell-autonomous regulatory pathways for tapetum development are depicted in the top orange box, and non-cell-autonomous signaling from/to the male germline are at the bottom. An upper half of the orange box shows the expression timing and biological functions of seven transcription factors (*TFs*), and a bottom half includes genetic pathways/events that take place in tapetal cells. In a top half, the cartoon symbols put within the white bars of each TF correspond to regulatory pathways and developmental events shown in a bottom half. In TF white boxes, gene names with cartoon symbols indicate direct targets of TFs in respective events, and the connections by dotted lines shows bHLH dimerization partners demonstrated in previous studies. A gray box connecting tapetum with male germline during meiosis represents the cytoplasmic channel (Heslop-Harrison 1966)

(*OsAM1*, Che et al. 2011). Analyses of multiple *am1* and *swi1* alleles unveiled a dual role of AM1 orthologs, once in meiotic entry and again in leptotene-zygotene progression in early meiosis I (Pawlowski et al. 2009; Merchier et al. 2001). Another factor for rice meiotic entry is MICROSPORELESS1 (MIL1), a plant-specific CC-type glutaredoxin (Hong et al. 2012).

Synchronous male meiosis in angiosperm microsporangia is important for simultaneous pollen production and efficient pollination, but the underlying mechanisms are largely unclear. The *Arabidopsis TARDY ASYNCHRONOUS MEIOSIS/CYCA1;2* gene, encoding an A-type cyclin, is required for synchronous transition from meiosis I to meiosis II (Wang et al. 2004). Rice MEL2 is a monocot-specific protein containing an RNA recognition motif and is involved in establishment of male synchrony (Nonomura et al. 2011). In vitro experiments suggest that MEL2 can target U-rich sequences, which are frequent in 3′-untranslated regions of hundreds of rice genes (Miyazaki et al. 2015). In *Drosophila*, maternal Nanos and Pumilio proteins binding to the 3′-untranslated regions of germline-specific mRNAs, including *Cyclin-B* mRNA, repress precocious cell division during migration of the germline to the gonad (Kobayashi et al. 1996; Asaoka-Taguchi et al. 1999), suggesting the importance of the cell cycle control mediated by RNA-binding proteins in proper germline development and meiotic entry in both animals and plants.

5.4 Meiosis-Specific Chromosomal Behavior

Prior to meiotic recombination and synapsis, epigenetic control of chromosomal behavior has an important role. Centromere association is thought to contribute to meiotic homologous recognition in meiosis. It takes place between not only homologous but also nonhomologous centromeres at leptotene, the onset of meiotic prophase I (Fig. 5.2a). In maize, the centromere association is promoted by STRUCTURAL MAINTENANCE OF CHROMOSOMES6 (SMC6) (Zhang et al. 2013). The association events and *SMC6* genes are conserved in diverse eukaryotic species, including rice (Zhang et al. 2013; Prieto et al. 2004). The loss of MEL1 Argonaute leads to failure to exit from centromere association (Liu and Nonomura 2016), implying that this process includes small RNA-mediated epigenetic pathways (see Sect. 5.7). Subsequently, telomeres are gathered around a limited region of the meiotic nuclear envelope, leading meiotic chromosomes to form a bouquet structure in many eukaryotes (Zickler and Kleckner 2016) (Fig. 5.2a). The meiotic bouquet is thought to facilitate homolog recognition, and is regulated genetically (Chikashige et al. 2006; Davis and Smith 2006), while the underlying mechanisms are largely unknown in rice. Bouquet formation in rice meiosis requires OsREC8 (Shao et al. 2011) and PAIR3 (Wang et al. 2011a, b), both of which are components of chromosomal axis (see Sect. 5.5), suggesting that proper recruitment of axial components couples with bouquet formation.

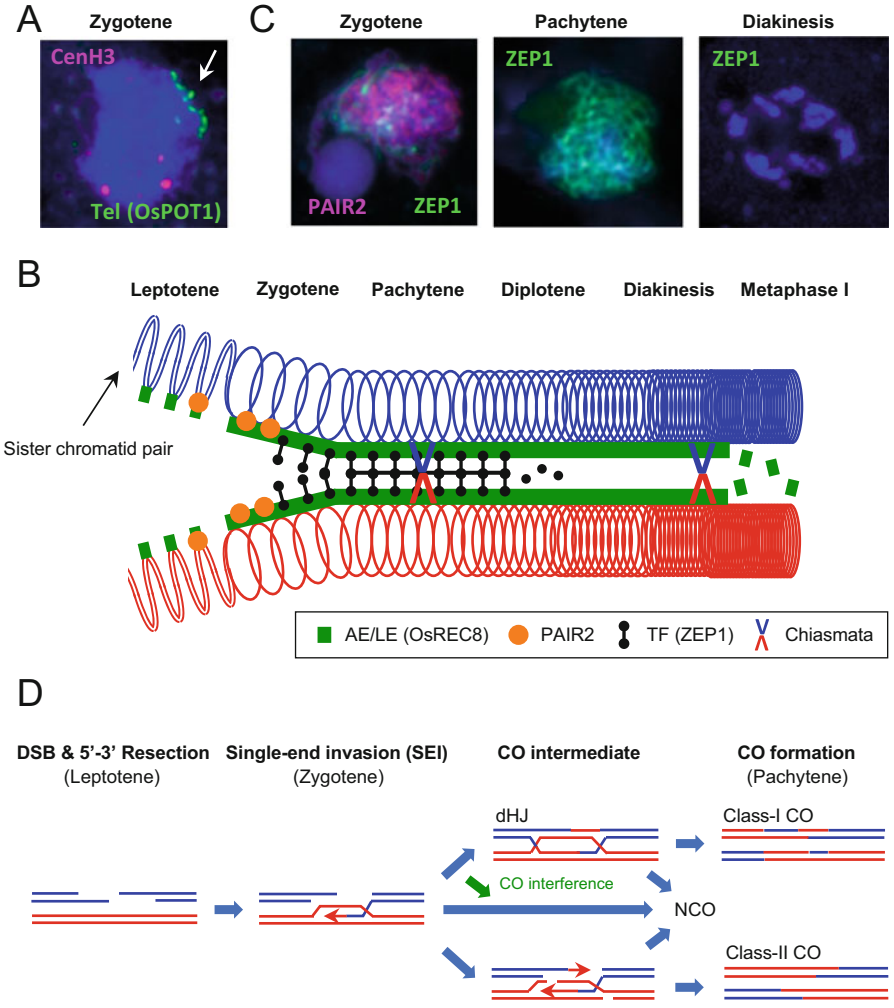

Fig. 5.2 Meiosis-specific chromosome behavior and homologous recombination. (**a**) A multicolor immunofluorescence of centromeres (magenta) and telomeres (green) in rice PMC nucleus at early zygotene. Chromosomes are counterstained with DAPI (blue). Homologous and nonhomologous centromeres are associated within foci in unequal sizes, and telomeres form "bouquet structure" (arrow). The photos in (A) and (C) are reuse of figures in Nonomura et al. (2011). (**b**) A schematic illustration of homologous chromosome synapsis in rice. The illustration is reuse of the figure in Burgoyne et al. (2009), modified for rice meiosis (Nature Publishing Group permission, No. 4093540691482). (**c**) SC formation during zygotene and pachytene in rice. PAIR2 proteins are removed from chromosomal axes where SC is established, and so, PAIR2 signals do not overlap ZEP1 signals during zygotene. (**d**) Meiotic DSB repair model proposed in budding yeast. The illustration is reuse of the figures in Heyer (2004) with modifications (Elsevier permission, No. 4100590757486)

5.5 Synaptonemal Complex Components

During leptotene, extension of axial elements (AEs) alongside each chromosome occurs concomitantly with meiosis-specific chromosome condensation (Fig. 5.2b) (Burgoyne et al. 2009). One of the major AE components is the cohesin complex, which is required for chromosomal architecture and sister chromatid cohesion. The cohesin complex is composed of four subunits: two SMC subunits and two non-SMC subunits (a kleisin subunit and a HEAT-repeat subunit) (Hirano 2006). Rice genome conserves four kleisin members: rice RADIATION SENSITIVE21-1 (OsRAD21-1) to OsRAD21-4 (Zhang et al. 2006). OsRAD21-4/MEIOTIC RECOMBINATION8 (OsREC8) prevents precocious separation of sister centromeres during meiosis I (Zhang et al. 2006; Shao et al. 2011). OsREC8 is located alongside entire meiotic chromosomes from leptotene to metaphase I and is supposed an important AE component (Fig. 5.2b) (Shao et al. 2011). In the *osrec8* mutant, thin threadlike decondensed chromosomes frequently appear during zygotene and pachytene, but are fully condensed by diakinesis. This finding suggests that the meiosis-specific condensation mechanism is separable from mitotic mechanisms and is coupled with AE formation. Importantly, loading of many proteins required for homologous synapsis and recombination (PAIR2, PAIR3, OsMER3, ZEP1; see Sect. 5.6 for respective protein functions) onto meiotic chromosomes depends on OsREC8 (Shao et al. 2011). Thus, AE provides a foundation for meiosis-specific events, such as meiotic recognition, recombination, and synapsis. HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1 (PAIR1) is a putative DNA-binding protein with coiled-coil motifs (Nonomura et al. 2004a). The *pair1* mutant shows similar meiotic phenotypes to *osrec8*, suggesting that PAIR1 is another AE component in rice. The rice coiled-coil protein PAIR3 is also an AE component required for homologous synapsis and meiotic recombination, but is dispensable for meiotic axis formation (Wang et al. 2011a).

During zygotene and pachytene, a ladderlike proteinaceous structure, called the synaptonemal complex (SC), is established between homologous pairs (Zickler and Kleckner 2016). In this process, AEs are included in SCs as lateral elements (LEs), and homologous LEs are connected by central components including numerous transverse filaments (Fig. 5.2b, c). ZEP1, the rice ortholog of budding yeast ZIP1 and *Arabidopsis* ZYP1, is a transverse filament component (Wang et al. 2010). CENTRAL REGION COMPONENT1 (CRC1), an AAA-ATPase orthologous to budding yeast Pachytene checkpoint2 (Pch2) and mouse *thyroid receptor-interacting protein13* THYROID RECEPTOR-INTERACTING PROTEIN13 (TRIP13), is another central component distinct from transverse filaments in rice (Miao et al. 2013). The *crc1* mutation affects meiotic chromosome condensation, similarly to *osrec8* and *pair1*, but differently from *zep1*, suggesting the CRC1 role in formation of AEs/LEs. Indeed, direct interaction of CRC1 with PAIR1 and ZEP1 was evident in a yeast two-hybrid assay (Miao et al. 2013). Pch2/TRIP13 promotes removal of HORMA (Hop1, Rev7, Mad2) domain protein from chromosomal regions, which is coupled with SC formation (Börner et al.

2008; Wojtasz et al. 2009). Similarly, PAIR2, the meiotic HORMA protein in rice (Nonomura et al. 2004b), is removed from AEs where homologous synapsis is established (Nonomura et al. 2006) (Fig. 5.2c). However, different from the animal system, CRC1 is rather required for initial loading of PAIR2 (Miao et al. 2013). P31^{comet} is recently identified as a protein putatively interacting with CRC1, and the loss of P31^{comet} leads to defects in meiotic DSB initiation and SC installation (Ji et al. 2016).

After SC destruction, OsREC8 still remains at centromeric regions to establish monopolar orientation of sister centromeres at metaphase I (Shao et al. 2011). Rice SHUGOSHIN1 (OsSGO1) prevents sister-centromere separation at metaphase I (Wang et al. 2011b, 2012b), probably via protection of OsREC8 degeneration. Rice BUB1-RELATED KINASE1 (OsBRK1) is orthologous to budding yeast Bub1, a central player in surveillance of improper spindle-kinetochore attachments and precocious segregation of sister kinetochores during meiosis I (Malmanche et al. 2006). OsBRK1 is required for centromere loading of OsSGO1 (Wang et al. 2012b).

5.6 Meiotic Recombination

Homologous recombination (HR) is initiated by programmed DSB formation, catalyzed by Sporulation-specific protein11 (Spo11), a subunit of the type II DNA topoisomerase (Keeney et al. 1997; Bergerat et al. 1997). The rice genome encodes five SPO11 paralogs, and OsSPO11-1 and OsSPO11-4 are functional in meiosis based on RNAi knockdown results (Yu et al. 2010; An et al. 2011; Shingu et al. 2012).

A key step in HR is to generate single-stranded DNA (ssDNA) at meiotic DSBs (Fig. 5.2d). In budding yeast, the Mre11-Rad50-Xrs2 (MRX) complex removes SPO11 from DSBs and produces 3′-overhanging ssDNA tails cooperatively with a DNA endonuclease, Com1/Sae2 (Alani et al. 1990; Cao et al. 1990; Nairz and Klein 1997; Tsubouchi and Ogawa 1998; Huertas et al. 2008). Replication protein A (RPA) is a multiprotein complex that protects ssDNA from exonucleases during DNA replication and DSB repair (Wold 1997; Iftode et al. 1999). In meiotic DSB repair, RPAs on ssDNA are replaced by bacterial RecA homologs, Rad51 and Dmc1, promoting the single-end invasion (SEI) into homologous double-stranded DNA (dsDNA) (Bishop 1994; Hunter and Kleckner 2001) (Fig. 5.2d). In addition to error-free HR, error-prone nonhomologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ) are known as SEI-independent DSB repair pathways (Daley et al. 2005; Lieber 2010; McVey and Lee 2008). Many of the proteins mentioned above are conserved in rice, and the loss or knockdown of OsMRE11, OsCOM1, and OsRPA1a/OsRPA70a all results in appearance of apparent meiotic chromosome fragmentation during meiosis I (Ji et al. 2012, 2013b; Chang et al. 2009), suggesting deficient DSB repair. Rice has five RAD51 and two DMC1 paralogs, which likely have diverse functions in HR (Lin et al. 2006; Byun and Kim 2014; Tang et al. 2014; Zhang et al. 2015; Wang

et al. 2016). The helical filaments of OsDMC1A and OsDMC1B extend over 5000 bp to promote strand exchange between ssDNA and dsDNA in the presence of RPA in vitro (Sakane et al. 2008).

After SEI, meiotic DSBs are repaired by two distinct HR pathways: class I and class II crossovers (COs) (Fig. 5.2d). In *Arabidopsis* and rice, the class I CO pathway accounts for ~90% of all COs (Higgins et al. 2004; Shen et al. 2012). In class I CO, after priming DNA synthesis using homologous strands, the 3′-end of a SEI is captured by the 5′-end (second-end capture), leading to formation of a double Holliday junction (dHJ), resolution of which results in either COs or non-COs (NCOs) (Szostak et al. 1983; Hunter and Kleckner 2001) (Fig. 5.2d). Hundreds of meiotic DSBs are formed in plants; however, the majority develops into NCOs (Mercier et al. 2005). In addition, class I COs repress secondary induction of nearby COs, which is called CO interference. On the other hand, at least one CO is assured in each homolog pair, referred to as CO assurance or obligate CO, for retaining homologous chromosome pairs until metaphase I. This CO homeostasis is indispensable for proper meiosis (Martini et al. 2006).

In budding yeast, formation of class I COs is mediated by ZMM proteins (Zip1, Zip2, Zip3/Mei4/Hei10, Zip4, Spo16, Msh4, Msh5, and Mer3) (Bishop and Zickler 2004; Börner et al. 2004). Non-interfering, ZMM-independent class II CO is mediated by MMS- and UV-sensitive protein81 (MUS81) and Methyl methanesulfonate sensitive4 (MMS4) (Boddy et al. 2001; Osman et al. 2003; Hollingsworth and Brill 2004; Schwartz et al. 2012). The rice genome conserves OsZIP1/ZEP1 (Wang et al. 2010), OsZIP4 (Shen et al. 2012), MUT-S PROTEIN HOMOLOG4 (OsMSH4), OsMSH5 (Luo et al. 2013b), OsMER3 (Wang et al. 2009), and HUMAN ENHANCER OF INVASION10 (OsHEI10) (Chelysheva et al. 2012; Wang et al. 2012a), all of which are class I CO components. OsGEN1 has Holliday junction resolvase activity in vitro, as do budding yeast Yen1 and human GEN1 (Yang et al. 2012), and is thought to act mainly in class II CO but also occasionally in class I CO generation in rice (Wang et al. 2017).

SC installation is closely related to class I CO formation. For example, loading of OsMER3 onto meiotic chromosomes depends on PAIR2, essential for SC installation (Wang et al. 2009). Elongation of SC central components is initiated at class I COs and is dependent on ZMM components in budding yeast (Agarwal and Roeder 2000; Fung et al. 2004). The number of ZMM protein foci corresponds to the number of class I CO in yeast. However, in *Arabidopsis* and rice, ZMM foci are in excess of the number of class I COs (Higgins et al. 2004; Shen et al. 2012). This inconsistency is probably due to ZMM proteins accumulating on pre-NCOs, in addition to pre-COs in plants (Shen et al. 2012).

OsRAD1 acts in NHEJ suppression, which is required for proper CO formation in rice (Hu et al. 2016), suggesting that strict control of the balance of error-free HR and error-prone NHEJ/MMEJ repair pathways is important for proper meiosis. OsHUS1 suppresses nonallelic homologous recombination or ectopic recombination (Che et al. 2014). OsRAD1 directly interacts with OsHUS1 (Hu et al. 2016).

5.7 Epigenetics in Meiotic DSB Initiation

In *Arabidopsis*, CO hot spots tend to overlap genic regions, especially around the transcriptional start sites, where nucleosome density is low and the histone variant H2A.Z is enriched (Choi et al. 2013), clearly indicating the importance of the epigenetic landscape in determining CO positions. The rice genome also has hot and cold spots for meiotic recombination (Si et al. 2015; Habu et al. 2015). *Arabidopsis* DECREASE IN DNA METHYLATION1 (DDM1) is a nucleosome-remodeling ATPase (Vongs et al. 1993; Jeddeloh et al. 1999; Brzeski and Jerzmanowski 2003). Rice has two DDM1 paralogs, OsDDM1a and OsDDM1b. The knockdown of both OsDDMs results in severe reduction of cytosine methylation in repetitive genomic sequences (Higo et al. 2012) and in an increase in recombination frequency and decrease in dimethylation of histone H3 lysine 9 (H3K9) at centromeric regions (Habu et al. 2015). A recent analysis unveiled that the epigenetic landscape of meiotic chromosomes is dramatically altered during meiotic entry in rice. This event, named large-scale meiotic chromosome remodeling (LMR), accompanies remarkably increased dimethylation and reduced acetylation of H3K9 through broad regions of chromatin (Liu and Nonomura 2016) (Fig. 5.3). The *mel1* mutant PMCs lack LMR, concurrent with the loss of meiotic DSB formation and SC installation (Liu and Nonomura 2016). All these findings imply that epigenetic manipulation of chromatin modifications can alter the frequency and position of meiotic recombination.

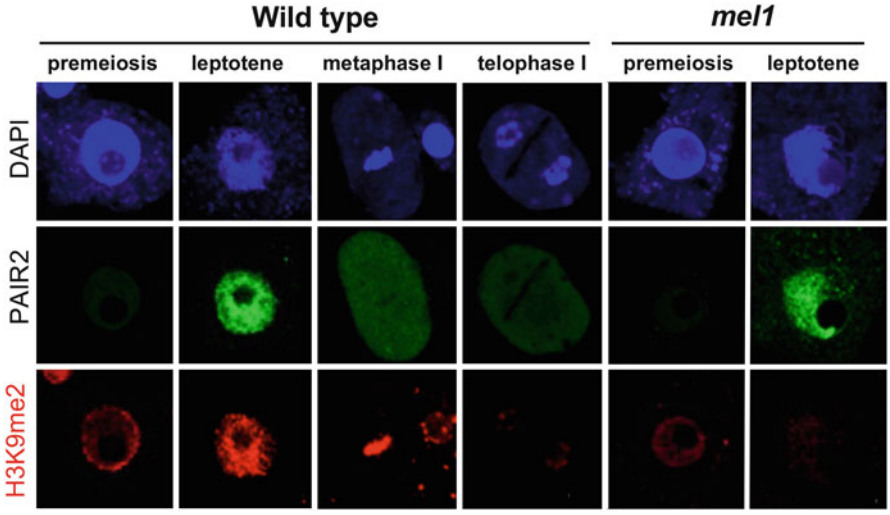

Fig. 5.3 Large-scale meiotic chromosome remodeling (LMR) in rice. The subnuclear localization of PAIR2 (green) and H3K9me2 (red) during rice meiosis I. In wild-type PMCs (left), the H3K9me2 level is significantly elevated during meiotic transition and is kept until metaphase I. This LMR fully depends on MEL1 function (right). The photos are reuse of those in Liu and Nonomura (2016)

5.8 Cell-Cell Communication in Meiosis and Postmeiosis

Somatic tapetal cells surrounding the male germline are required for non-cell autonomous regulation of proper meiosis progression. In the *msp1* mutant, malformation of anther walls results in serious delay of male meiosis (Nonomura et al. 2003). MEL2 and MIL1, which regulate proper meiotic entry, are expressed in both germ cells and somatic anther wall cells (Nonomura et al. 2011; Hong et al. 2012), also implying the importance of cell-cell communication in anthers. Though no intercellular mobile signal has been reported in rice meiotic anthers, small RNA is a strong candidate. Phased small interfering RNA (phasiRNA) is a unique small RNA class in plants (Fei et al. 2013). The 21-nucleotide (nt) phasiRNA is abundant in premeiotic anthers, and the 24-nt phasiRNA is expressed in meiotic tapetum in maize (Zhai et al. 2015) and rice anthers (Fei et al. 2016). MEL1 can associate with both premeiotic 21-nt and meiotic 24-nt phasiRNAs, in addition to 24-nt repeat-associated siRNAs, in germ cells (Komiya et al. 2014). Recently, it has been revealed that a subset of 24-nt MEL1-associating RNAs in PMCs is originated from 24-nt tapetum-originating phasiRNAs, suggesting the cell-cell mobile nature of 24-nt phasiRNAs (Ono et al. 2018). This finding is consistent with a previous report in maize that isolated PMCs contain considerable numbers of 24-nt phasiRNAs (Dukowic-Schulze et al. 2016). Downstream events of the MEL1 pathway involve LMR including changes in H3K9 dimethylation (Liu and Nonomura 2016) (Fig. 5.3), implying a role of nuclear MEL1/24-nt RNAs in modifications of chromatin required for meiotic recombination and homologous recognition.

Lipid metabolism and secretion are central roles of the tapetum in postmeiotic non-cell autonomous regulation that allows construction of the outer pollen wall, called exine. During meiosis I, tapetal cells are binucleated in rice, probably to specialize in production of nutrients and materials for development of PMCs and microspores. Precursors of sporopollenin, which makes up the bulk of modified lipid molecules insoluble in both aqueous and organic solvents, are secreted from tapetal cells, and extracellular granules of sporopollenin, called Ubisch bodies, are supplied to form exine (Fig. 5.1c). The plastid-derived fatty acid precursors of Ubisch bodies undergo multiple metabolic processes mediated by a series of catalytic enzymes, such as fatty acid reductases, fatty acyl-CoA synthases, and cytochrome hydroxylases (Jung et al. 2006; Li et al. 2010, 2016; Shi et al. 2011; Yang et al. 2014, 2017). Ubisch bodies also carry nutrients and proteins to microspores (Wang et al. 2003). Rice ATP-BINDING CASSETTE G TRANSPORTER15 (ABCG15) is preferentially distributed on the microspore side of tapetal cell membranes to selectively transport sporopollenin precursors and other molecules into anther locules (Niu et al. 2013a; Wu et al. 2014; Zhao et al. 2015). C6 also acts in sporopollenin transport in rice (Zhang et al. 2010). In contrast to tapetum-secreted pollen constituents, MICROSPORE AND TAPETUM REGULATOR1 (MTR1), a fasciclin glycoprotein, is secreted from PMCs and microspores into the anther locule and acts as an intercellular signal between the male germline and somatic tapetum (Tan et al. 2012).

5.9 Tapetal PCD

PCD-assisted degradation of tapetal cells is indispensable for recycling cell materials into pollen coat constituents (Jessen et al. 2011). CP1 is the initial signal inducing tapetal PCD (Lee et al. 2004). Two rice aspartyl proteases, AP25 and AP37, putative analogs of the caspase, involved in animal apoptosis, are also important for tapetal PCD induction (Niu et al. 2013b). APOPTOSIS INHIBITOR5 (API5) is another PCD inducer interacting with the DEAD-box ATP-dependent RNA helicases, API5-INTERACTING PROTEIN1 (AIP1), and AIP2 (Li et al. 2011b).

Reactive oxygen species (ROS) regulate cell death events in plants (Overmyer et al. 2003), and ROS accumulation is closely related to tapetal PCD in rice. Rice DEFECTIVE TAPETUM CELL DEATH1 (DTC1) triggers expression of the PCD-inducible protease genes C*P1*, *AP25*, and *AP37* and is a key regulator promoting ROS accumulation and PCD in the tapetum (Yi et al. 2016). DTC1 suppresses the activity of the ROS-scavenger METALLOTHIONEIN2b (MT2b) by direct interaction. The mitochondrial protein CYTOCHROME C OXIDASE11 (COX11) acts in peroxide metabolism and prevention of abnormal ROS bursts of tapetal cells during male meiosis. A natural variation in *WILD ABORTIVE352* (*WA352*), a mitochondrial gene of wild relatives of rice, inhibits COX11 activity and leads to a premature tapetal ROS burst and PCD, resulting in wild-abortive cytoplasmic male sterility (CMS-WA) (Luo et al. 2013a).

Autophagy is an intracellular self-degradation and metabolite recycling system widely conserved in eukaryotes. Rice AUTOPHAGY7 (ATG7) is a ubiquitin-activating E1-like enzyme, and ATG9 is a transmembrane protein. Both are central regulators in autophagosome formation and tapetum degeneration (Kurusu et al. 2014). Interestingly, there is no autophagy-defective mutant reported to be male sterile in *Arabidopsis* or maize (Yoshimoto 2012; Hanamata et al. 2014; Li et al. 2015), suggesting that rice may have evolved a unique autophagy-assisted system in tapetal PCD.

5.10 Pollen Polarization and Asymmetric Pollen Mitosis I

Rice pollen is a nearly globular shape with a single pollen aperture on the exine wall (Fig. 5.4a), and aperture formation is a key for cell polarity determination in pollen development. The microspore nucleus migrates to the antithetical side of the aperture followed by pollen mitosis I (PMI), and a small generative cell is integrated into the cytoplasm of a larger vegetative cell (Zhang et al. 2005) (Fig. 5.4a). The generative cell, a precursor of sperm cells, is also characterized by compacted nuclear chromatin, in contrast to the vegetative cell, which has dispersed nuclear

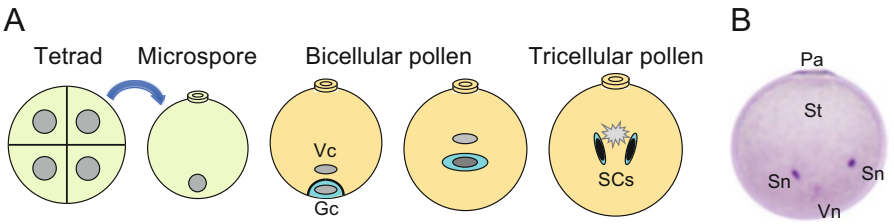

Fig. 5.4 Pollen mitosis I and maturation in rice. (**a**) A pollen tetrad is produced via meiosis and is divided into four microspores. The microspore nucleus migrates to the antithetical side of the pollen aperture. The microspore undergoes asymmetric mitosis to yield a large vegetative cell (Vc: tangerine) and a small generative cell (Gc: blue). The generative cell is incorporated into the vegetative cytoplasm and produces two sperm cells (SCs) via symmetric mitosis II. (**b**) The matured pollen grain stained with 1% hematoxylin solution. Just before anthesis, starch granules (St) are distributed to the pollen aperture side, while two condensed sperm nuclei (Sn) and a dispersed vegetative nucleus (Vn) are observed at the opposite side to the pollen aperture (Pa)

chromatin specified for pollen tube germination and elongation. After bicellularization, the vegetative cell begins to produce starch granules in the cytoplasm. The granules are evenly distributed through pollen development. However, in tricellular pollen just before anthesis, they are redistributed to the aperture side quickly (in <24 h) for subsequent tube elongation. Concomitantly, the vegetative nucleus migrates to the opposite side with two sperm cells (Fig. 5.4b).

PMI is a typical asymmetric cell division. In *Arabidopsis* PMI, GEMINI POLLEN1 (GEM1), GEM3, and TWO-IN-ONE (TIO) are essential to establish cell polarity and/or cytokinesis. GEM1 is a microtubule (MT)-associating protein (Twell et al. 2002). GEM3 is involved in MT-dependent MT nucleation in acentrosomal cells (Oh et al. 2005). TIO is a phragmoplast MT-associating kinesin motor protein (Oh et al. 2016). All these genes are conserved in the rice genome (see below) and are expressed in both pollen grains and somatic tissues (Hotta et al. 2012; McMichael and Bednarek 2013). Collectively, in addition to reproduction-specific genes, mitotic apparatus proteins drive asymmetric PMI.

Gene transcription and chromatin structure are strictly and spatiotemporally regulated during cell differentiation after PMI. SIDECAR POLLEN (SCP)/LATERAL ORGAN BOUNDARIES27 (LBD27) and LBD10 are TF-like proteins that can form a heterodimer and can establish cell polarization and patterning during PMI (Oh et al. 2010; Kim et al. 2015). LBD10 interacts with other LBD proteins in vegetative cells (Kim et al. 2016). *Arabidopsis* DUO POLLEN1 (DUO1) is an R2R3-type MYB TF, required for differentiation of the generative cell into sperm cells (Rotman et al. 2005; Brownfield et al. 2009). REGULATORY REGION OF DUO1 (ROD1) has been identified as a *DUO1* activation factor (Peters et al. 2017). During generative cell division and differentiation, special types of histone variants are thought to help condensed chromatin structures specific to sperm cells in *Lilium* (gH2A, Ueda et al. 2012) and *Arabidopsis* (MGH3, Okada et al. 2005).

5.11 Pollen Maturation and Germination

After PMI, the generative cell is detached from the vegetative cell wall and the pollen is enlarged. These processes require active metabolism to produce pollen cell walls. Rice GLYCOSYLTRANSFERASE1 (OsGT1) is essential for formation of intine, the internal pollen coat component (Moon et al. 2013). COLLAPSED ABNORMAL POLLEN1 (CAP1) is an L-arabinokinase responsible for wall formation in generative and vegetative cells (Ueda et al. 2013). The importance of L-arabinose metabolism in pollen development is also suggested from functional analysis of UDP-ARABINOPYRANOSE MUTASE3 (OsUAM3) (Sumiyoshi et al. 2015). RICE IMMATURE POLLEN1 (RIP1), a WD40-repeat protein, promotes pollen development at the bicellular and later stages (Han et al. 2006).

After pollination, rice MILDEW RESISTANCE LOCUS O12 (OsMLO12), which has seven transmembrane domains, is required for pollen hydration during pollen tube germination (Yi et al. 2014). Inorganic ions, such as Ca^{2+} and K^{+}, play essential roles in tube germination and elongation (Holdaway-Clarke and Hepler 2003). Rice RUPTURED POLLEN TUBE (RUPO), a plant-specific receptor-like kinase that interacts with potassium transporters, represses precocious bursting of elongating pollen tubes (Liu et al. 2016). OsBOR4 is a pollen-specific efflux transporter of boron (Tanaka et al. 2013), which is required for structural maintenance of cell walls (O'Neill et al. 2004). The loss of OsBOR4, SUCROSE TRANSPORTER1 (OsSUT1) (Hirose et al. 2010), OsAP65 (Huang et al. 2013), or IMPORTIN β1 (OsIMOβ1) (Han et al. 2011) reduces the ratio of inheritance via male gametes, but has little impact on pollen morphogenesis. These results all imply the existence of complicated metabolic networks from pollination to fertilization, including the transport of boron and sucrose, proteolysis, and nuclear protein imports.

5.12 Application of Knowledge from Plant Reproduction Studies

Manipulation of the frequency and positions of meiotic recombination is one of the final goals to improve efficiency of conventional breeding methods. Achieving this, however, requires unveiling many of the genetic and epigenetic mechanisms underlying meiosis. What is possible based on current knowledge is quite far from the goal, especially related to epigenetics.

Breaking through interspecific barriers in reproduction is another important objective to introduce natural genetic variations into modern cultivars. Hexaploid bread wheat is composed of three different genomes (Borrill et al. 2015). The wheat gene *PAIRING HOMOEOLOGOUS1* (*Ph1*) suppresses pairing of homoeologous counterparts, which contribute to diploid-like chromosomal behavior (Moore 2014; Koo et al. 2016). The *ph1* mutation can be used to promote introgression of

non-wheat genes into the wheat genome (Rey et al. 2015). Interestingly, the mutation affects centromere association (Martinez-Perez et al. 2001), clearly indicating that further studies of epigenetic mechanisms are necessary for development of efficient breeding methods.

Applied use of mutations in reproductive processes and meiosis is being tried in model plants. For example, mutation of genes suppressing non-interfering class II CO increases the total number of COs in *Arabidopsis* (Crismani et al. 2012). Another example is the *Arabidopsis* triple mutant *atspo11*/*atrec8*/*osd1*, which displays apomeiosis (d'Erfurth et al. 2010), a mode of apomixis that converts meiosis into mitotic division (Grimanelli et al. 2001). The triple mutant can be used for production of clonal offspring and fixation of superior traits in hybrid vigor (Spillane et al. 2004), but in its application, a parthenogenetic trait must be added to the mutant, otherwise the ploidy level is doubled at every fertilization (d'Erfurth et al. 2010). In rice, the *pair1*/*osrec8*/*ososd1* triple mutant can be used for the same purpose (Mieulet et al. 2016). In pollen development, cytoplasmic male sterility due to genetically disturbed nuclear-mitochondrial interaction is used for commercial development of hybrid lines (Wilson and Zhang 2009). However, we are just standing at the gate for development of new breeding technologies using meiosis- and reproduction-related genes.

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Chapter 6
Transcriptome Dynamics in Rice Leaves Under Natural Field Conditions

Takeshi Izawa

Abstract Although crops have been domesticated and bred under natural field conditions, the majority of molecular genetic analyses have been performed under controlled artificial conditions, such as growth chambers. This restricts agricultural application of new findings on important crops based on molecular genetics. Recently, several transcriptome analyses to elucidate the dynamics of the transcriptome and several specific biological traits have been reported. These analyses made full use of cutting-edge methods of statistical modeling and Bayesian approaches. One critical finding of these studies was that thousands of genes expressed in rice leaves respond significantly to dynamic changes in ambient temperatures under natural fluctuating conditions. This should serve as a wake-up call for plant researchers using fixed-temperature conditions in growth chambers. This chapter discusses the processes involved and provides longitudinal perspectives on field transcriptome analysis.

Keywords Field transcriptome · Fluctuating environments · Statistical modeling · Crop science · Molecular biology

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6.1 Reasons Why Plant Molecular Genetic Researchers May Be Hesitant to Perform Experiments Under Field Conditions

6.1.1 Dealing with Fluctuating Data Obtained Under Natural Conditions Using Statistical Analysis

Many researchers hesitate to use data obtained under natural field conditions because the reproducibility of experimental data would be severely affected under fluctuating ambient growth conditions. In contrast, experimental data are easily reproducible under artificially controlled growth conditions in the laboratory, so researchers do not have to repeat experiments to obtain statistically significant results. Molecular geneticists generally attempt to minimize the repetition of experiments, which is appropriate when working on mutant lines exhibiting very clear genetic phenotypes. In such cases, fold changes and differences between wild-type and mutant plants are normally high. However, when analyzing complex genetic traits, known as quantitative trait loci (QTL), experimental data contain both genetic variations due to segregation and environmental variations due to differences in growth settings under natural field conditions (Lander and Botstein 1989). Thus, QTL analyses include statistical evaluations, such as logarithm of odds (LOD) scores, to select the next approach or target locus. Furthermore, to evaluate agronomic traits of a new variety, many crop breeders normally grow a number of candidate lines for several years in at least a dozen areas. Therefore, upon next-genome sequencing era, not only plant breeders but also plant molecular biologists should become familiar with experiments on this scale. Statistically significant results obtained on a small scale with a small number of experiments are not suitable for practical use.

To examine the significance of transcriptome data, it has been recommended to use criteria based on multiple testing, such as the false discovery rate (FDR) (Yang et al. 2003). However, we often obtain only a few genes which can be beyond such criteria significantly with several repeats of transcriptome analyses. In our experience, the paired *t*-test using 40 pairs of rice leaf transcriptome data yielded several thousand genes that showed statistically significant changes in expression even after FDR correction (Izawa et al. unpublished). Interestingly, all of these paired samples were sampled at distinct timings, although they were obtained from two neighbor paddy fields with and without nutrition. Furthermore, most of the significant genes among the 40 pairs of transcriptome data exhibited very small fold changes of mean values in expression. Meanwhile, hundreds of genes showed significant changes in expression when we used 20 pairs of transcriptome data. These observations suggested that statistical analyses with more than a dozen transcriptome data would give us satisfactory results, even when there is a great

deal of experimental noise due to differences in environmental conditions. In addition, it might be very important that the fold changes should be taken into consideration rather than FDR-corrected *P*-values to select biologically important genes. That is, even the statistical significance obtained with larger amounts of data may have limits for identifying the biological importance.

6.1.2 Correlations Among Distinct Environmental Factors

Temporal correlations between distinct environmental factors in a few days, such as solar radiation and ambient temperature, are often so high and too difficult to distinguish them on which factors were relevant to the studies of plant biology. However, such correlations between distinct environmental data differ between seasons. Thus, we found previously that temporal correlations between the fluctuations in various environmental data over several months relatively become small (Izawa 2015). Therefore, when considering all of the crop seasons of target crops over several years, the correlations among environmental factors are thought not to be an obstacle for further biological analysis.

6.1.3 Costs of Gathering Field Transcriptome Data

When planning field transcriptome analysis, it is necessary to consider the costs associated with gathering data. As of 2016, the financial costs to perform transcriptome analysis are around 30,000 Japanese yen per sample. Here, we used a custom microarray with 180 K probes provided by Agilent Technologies. In a series of preliminary experiments, we examined the use of Cy5 and Cy3 labeling for the same RNA samples. After calibration to correct for characteristic trends between Cy5 and Cy3 signals using software provided by the manufacturer, we found that there were no differences in data according to which label was used (Izawa et al. data not shown). Thus, we do not care about the swapping effects between Cy5 and Cy3 for the cost efficiency in our field transcriptome analysis. Although the numbers of transcriptome data required for biologically significant field transcriptome analysis depend on the purpose of individual research, around 100 samples for one class of data would be sufficient for most purposes. We usually perform analyses using 12–13 samples per day to obtain the diurnal changes of transcriptome.

In addition, the major matters to be attended carefully to obtain reliable good quality data are those associated with sampling, RNA preparation, labeling, and microarray analysis. For sampling, we use standard tubes for all collaborating

teams and provide guidelines for sampling in the field. For microarray analysis, we use an open laboratory facility at the National Agriculture and Food Research Organization (NARO) in Tsukuba, Japan. In this open laboratory, experts in handling RNA and in operation of microarray equipment support us well to generate very reliable microarray data for all of our samples.

The RNA sequencing (RNA-Seq) technique provides a great deal of information for each transcript, such as its initiation site or splicing variants. We compared microarray analysis and RNA-Seq data for the same RNA samples and found that both yielded comparable data. Furthermore, we found that the reliability of transcriptome data between RNA-Seq and microarray data differed among target genes. Thus, it is difficult to say which of the two is better at this moment. In addition, the financial costs associated with both methods to obtain comparable data are currently almost the same. However, primary analysis of raw RNA-Seq data using a high-performance computer is still more laborious and complicated than microarray analysis. In addition, the conversion of legacy microarray data to the corresponding RNA-Seq data is largely dependent on the platform used and requires the setting of distinct rules for data regarding each gene. In the near future, direct RNA-Seq, such as the MinION nanopore sequencer technology, should be considered in place of the microarray method.

6.2 Environment-Driven Statistical Modeling of the Transcriptome

6.2.1 Lognormal Distribution Assumption for Transcriptome Data

For statistical modeling, experimental noise should be distributed according to a normal distribution. In contrast, many raw data related to gene expression, including qRT-PCR data and transcriptome data obtained by microarray analysis, are not normally distributed. Empirically, it is known that qRT-PCR and transcriptome data obtained by microarray analysis have a lognormal distribution (Izawa 2012). Therefore, all transcriptome data should be transformed in a logarithmic manner before further statistical analysis. It is better to consider transforming data to a lognormal distribution when raw data obtained with larger values possesses larger experimental standard deviations. Experimental noise may not depend on the range of measured data.

6.2.2 A Model of Field Transcriptome Analysis in Rice

Determining the drivers of gene expression patterns is more straightforward under laboratory conditions than in the complex fluctuating environments seen in the field. Nagano et al. (2012) reported gathering 461 transcriptome data from the leaves of rice plants in a paddy field along with the corresponding meteorological data and developed statistical models for the endogenous and external influences on gene expression. In total, expression dynamics of more than 20,000 genes could be explained based only on information regarding environmental conditions, such as sampling date and time, transplantation date, and meteorological environmental data (solar radiation, temperature, atmosphere, wind, and precipitation). The results indicated that transcriptome dynamics were predominantly governed by endogenous diurnal rhythms, ambient temperature, plant age, and solar radiation. The data revealed diurnal gates for environmental stimuli to influence transcription and pointed to relative influences exerted by circadian and environmental factors on different metabolic genes.

6.2.2.1 Pretreatment of Raw Environmental Data

Gene expression data are likely to be influenced by the dynamics of environmental factors. To integrate information regarding past dynamic changes in the environment, we pretreated environmental data with both a prefixed threshold for perception and a prefixed perception period. We prepared a series of pretreated environmental data with all possible combinations of several prefixed thresholds and several prefixed periods and tried to the best combination to explain the dynamic patterns of gene expression. Thus, we were able to test various perception patterns of dynamic data from each environment from a single set of temporal environmental data. With this approach, we can evaluate which environmental factors contribute to the dynamics of target genes.

6.2.2.2 Grid Search Modeling of the Transcriptome

To select the best among various pretreated environment data, we developed a linear model that connects several terms, including bias, development, clock, and environment, to explain the dynamics of gene expression in the field. Among the candidate models, the best was selected by a grid search. Six environmental factors were considered: solar radiation, ambient temperature, humidity, atmosphere, wind, and precipitation for the entire crop season. Each environmental factor was considered on a distinct grid, with each grid location having prefixed thresholds and periods of perception. In addition, we considered gate effects, which reflect the diurnal changes of sensitivity for each environmental factor. Several hundred thousands of grid locations were evaluated to select the best model. In fact, we

developed models for 21,173 genes in which the dynamics of gene expression can be explained by the environmental conditions among 23,000 genes expressed in rice leaves under field conditions during the crop season. We considered a model successful when the residual values between observation and prediction (or estimation) were normally distributed. Several hundred genes expressed in rice leaves were still not modeled with our method, suggesting that other environmental factors, such as abiotic and biotic stress, are required to explain the dynamics of these genes.

6.2.2.3 Major Findings from This Model

One of the most important findings in this modeling analysis was that more than 3000 genes are affected by the history of ambient temperature with specific thresholds and perception periods. As most plant researchers grow their research plant materials under artificial conditions with a fixed temperature, the findings of such studies are not necessarily reproducible at different temperatures. On the other hand, around 3000 genes are expressed very stably in rice leaves and are not affected by environmental or developmental factors. Of course, this model also generated predictions for the influence of changing temperature on transcriptome dynamics (Fig. 6.1). The models would also help to translate the knowledge amassed in laboratories to problems in agriculture, and our approach to deciphering the transcriptome fluctuations in complex environments will be applicable to other organisms.

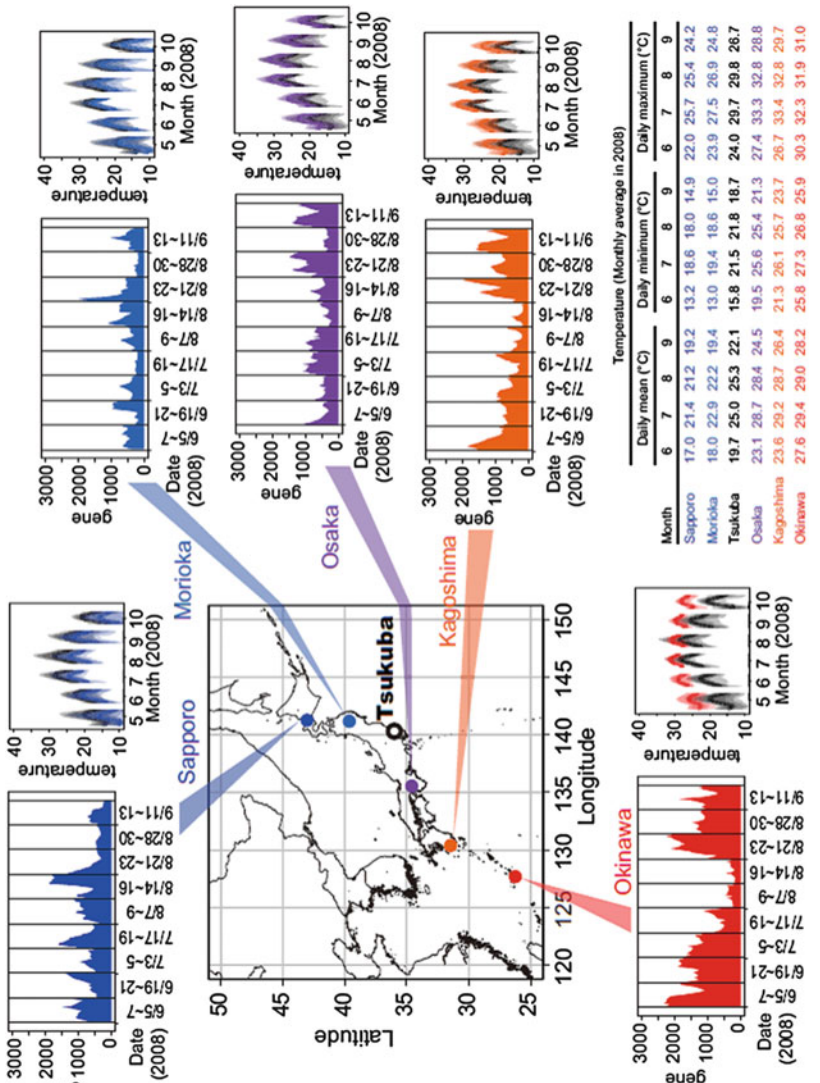
6.2.3 Differential Equation-Based Modeling of the Transcriptome

In the above model (Nagano et al. 2012), the degree of generalizability to predict the entire transcriptome is quite high with Pearson's correlation coefficients of 0.95 between observations and predictions of the transcriptome based on environmental data. However, the generalizability for half of the genes was still not practical to predict gene expression based only on environmental data. One major reason for this is that we attempted to explain gene expression based on only one environmental factor. Many genes are likely to be regulated by several environmental factors, such as light and temperature signals. However, we selected the most effective environmental factor among the six tested—solar radiation, temperature, humidity, atmosphere, wind, and precipitation. To improve the generalizability of genes that did not show practical abilities, it is necessary to simultaneously integrate information from at least two independent environmental factors to explain the expression of the target gene. Therefore, we developed a new method to develop such models using a differential equation to input two temporal environmental

factors (Matsuzaki et al. 2015). From the data of the previous model, we focused on solar radiation and ambient temperature to explain the expression of a set of genes involved in the circadian clocks in rice. Here, we did not attempt to develop models at the transcriptome level as the calculation cost to determine the appropriate parameters would become much larger than the simple grid search models. In the previous case, we utilized the nonlinear least squares method to determine the parameters as algebraic solutions for each grid. We then attempted to select the grid to fit the gene expression data. In this case, we used the particle swarm optimization method, where several particles that form a vector of numerical parameter values are dispersed in the parameter space to search for optimal values of each parameter (Lu et al. 2002). The numerical values of each particle are updated after consideration of the inertia of each particle, the center of balance of all of the particles, and the direction to the optimal position. Therefore, they are not independent but are weakly connected to each other. With updated particle values, the differential equation is then solved again, and the values are evaluated for the next update. With this system, we can perform machine learning to determine the average values of all particles as the best parameters after iterated learning processes. The new model obtained in this way clearly improved the generalizability compared to the previous model selected by the grid search for most of the 20 circadian clock-related genes in rice examined in this study. We recently developed a fast algorithm to perform this modeling at the transcriptome level using an improved ABC (approximate Bayesian computation) method for parameter regression (Lenormand et al. 2013). We are currently preparing a two-environmental factor-driven model of the transcriptome with this algorithm.

6.2.4 Potential Use of the Neural Network Algorithm Concept for Transcriptome Modeling

Although we have not yet evaluated the above two-environmental factor model, in the near future, we will consider the synergistic interactions of two environmental factors to explain gene expression for a special group of genes that are very sensitive and exhibit complicated responses to a given environment. To construct such a model, it could be reasonable to utilize the concept of multiple neural network algorithms (LeCun et al. 2015). This algorithm is known to be sufficiently flexible and rich to explain complicated interactions of various inputs. To maintain generalizability, we should use regularization terms in squared loss function and/or cross-validation. In addition, only a few pretreated inputs of environmental factors and pretreated time information should be used to make a model. Then, the best model among distinct combinations of pretreated data would be selected to obtain novel knowledge in biology. However, it is still not clear how many genes will be targets in a model with such complex interactions among environmental factors.



Temperature (Monthly average in 2008)

Month	Daily mean (°C)				Daily minimum (°C)				Daily maximum (°C)			
	6	7	8	9	6	7	8	9	6	7	8	9
Sapporo	17.0	21.4	21.2	19.2	13.2	18.6	18.0	14.9	22.0	25.7	25.4	24.2
Morioka	18.0	22.9	22.2	19.4	13.0	19.4	18.6	15.0	23.9	27.5	26.9	24.8
Tsukuba	19.7	25.0	25.3	22.1	15.8	21.5	21.8	18.7	24.0	29.7	29.8	26.7
Osaka	23.1	28.7	28.4	24.5	19.5	25.6	25.4	21.3	27.4	33.3	32.8	28.8
Kagoshima	23.6	29.2	28.7	26.4	21.3	26.1	25.7	23.7	26.7	33.4	32.8	29.7
Okinawa	27.6	29.4	29.0	28.2	25.8	27.3	26.8	25.9	30.3	32.3	31.9	31.0

Fig. 6.1 Prediction of gene expression under different growing conditions. Predicted changes in gene expression in five locations at different latitudes in Japan based on environment-driven statistical modeling of the transcriptome (Nagano et al. 2012). For each location, the left plot indicates the number of genes predicted to differ in expression by more than twofold relative to corresponding data from Tsukuba, Japan. The *horizontal axis* represents the time points. The right plot indicates diurnal temperature data (00:00–24:00) for each month at that particular location (colored) and Tsukuba (black). Thick lines represent averages. The area densities represent ranges containing 90, 75, and 50% of data. The lower-right table shows monthly averages of the daily mean, minimum, and maximum temperature in 2008 (Cited from Fig. 7 in Nagano et al. 2012)

6.3 Transcriptome-Driven Sparse Modeling of Biological/Agricultural Traits

6.3.1 Bayesian Filtering Using Two-Dimensional Probability Distribution Heat Maps

Transcriptome data obtained under field conditions is likely to contain enough information to explain dynamic changes in biological and agricultural traits (Yang et al. 2011). One simple way to extract such information on various traits is to determine the two-dimensional relationships of probability between the target trait and expression of a gene within a cluster of genes. According to Bayes' theorem, with information regarding gene expression in a set of selected genes, the target trait can be narrowed down as a probability distribution (Matsuzaki et al. 2015). In this way, we demonstrated that having gene expression data of only 16 related genes was sufficient to estimate the time of sampling with an accuracy of around 20 min (Figs. 6.2 and 6.3). Here, we examined 25 circadian clock-related genes and selected 16 genes. We searched all possible combinations of the 25 genes to select the best combination. This way works well for circadian clock-related traits since we were able to focus a dozen genes before selecting the best combination. However, this indicates that this way is not possible when we have transcriptome data, which includes more than ten thousand data, to select related genes.

6.3.2 Sparse Modeling of the Transcriptome with Regularization Terms, Such as LASSO Regression

To find any relationship between gene expression of individual genes in transcriptome and any biological/agricultural traits, it would be a good way to develop a sparse modeling method to explain biological traits using gene expression data as the input. Here, it is important to extract as many genes related to biological traits as possible and establish statistical models to predict the traits with high generalizability. We examined the use of least absolute shrinkage and selection operator (LASSO) regression methods to develop such a model (Tibshirani 1996). Although we were unable to develop such models for various traits using transcriptome data artificially randomly mixed and connected to trait data, we were able to establish practical LASSO models for various traits with transcriptome data appropriately connected to trait data. Thus, the LASSO method is a very promising way. As we used glmnet, which provides the final model after tenfold cross-validation, the generalizability of the obtained model was sufficient. We used hundreds of microarray data containing 20–50 K data of gene expression to make

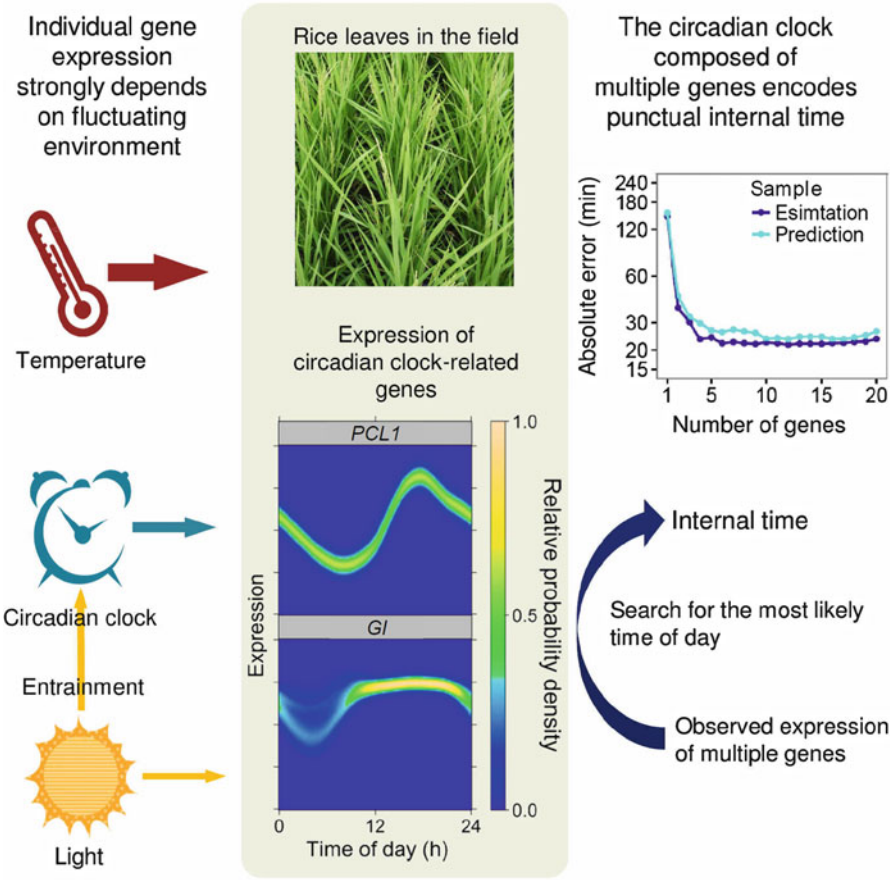


Fig. 6.2 Construction of transcriptome-driven model for trait prediction**.** Left: a model of individual gene expression in wild-type rice growing in the field responding to solar radiation and ambient temperature was built for circadian clock-related genes and indicated a strong effect of temperature. Middle: prediction by the model was used to determine the relationship between physical time of day and expression. Right: using the relationship, we can infer internal time from expression of multiple genes and found accuracy to 22 min relative to physical time regardless of weather, day length, or plant developmental age (Cited from Supplemental Fig. 6.1 in Matsuzaki et al. 2015)

such LASSO models, and so we often obtained around 100 genes to make a LASSO model. Expression data of most of the selected genes showed clear correlations with the target trait values, but several genes did not. Thus, some synergistic interactions between gene expression values were integrated into the LASSO model. However, it should be noted that not all of the genes in the obtained LASSO model reflect all of the genes related to the trait. Thus, a new method is needed to extract all of the genes related to the target trait.

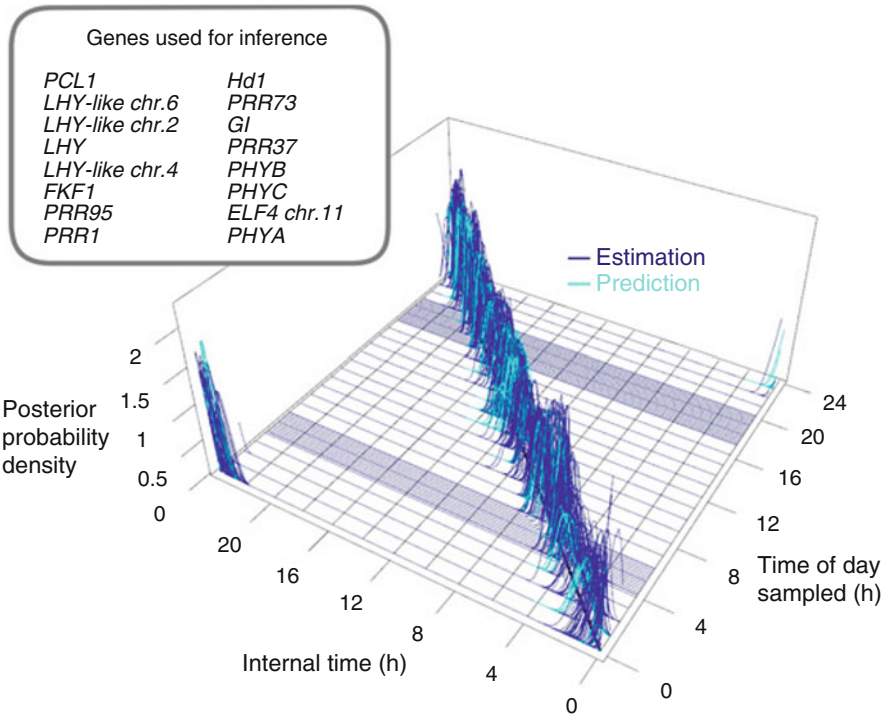


Fig. 6.3 Prediction of sampling time based on expression data of 16 circadian clock-related genes. Estimation and prediction of sampling time using gene combination with the best estimation performance (i.e., the lowest mean absolute estimation). Posterior probability density of predicted sampling time is plotted against time of day sampled. Each blue (training sample for estimation, $n = 461$) and turquoise (validation sample for prediction, $n = 125$) line corresponds to a single sample. Among the training samples, those obtained at 10-min intervals at 04:00–06:00 and 17:00–20:00 are included. Ranges of predicted sampling time with zero posterior probability density for those samples are presented as areas with dense blue lines at the bottom of the three-dimensional (3D) space. The thick black diagonal line at the bottom of the 3D space indicates correspondence between internal time and time of day sampled. (Cited from Fig. 3 in Matsuzaki et al. 2015)

6.4 Potential Use of Field Transcriptome Data to Check Crops

In our preliminary LASSO model developed for prediction of heading date from sample RNAs (Izawa et al. data not shown), the accuracy of heading date prediction was around 2 days. Unlike the typical phenology model used to predict the target trait (Nakagawa et al. 2005), in which both real historical and predicted future meteorological data were needed, the LASSO model used only a single RNA sample to predict future traits. It is likely that such RNA may contain the developmental status of the sample and can therefore be used to predict the future from such

samples. This type of model would provide information regarding related genes, which can be used as biomarkers to predict biological traits. In addition, such RNA contains information on the health status of crops in the field, and LASSO modeling enables us to narrow the transcriptome data down to several key genes to find practical biomarkers to estimate the health status of crops. Applying this method to plant disease responses may provide information regarding the appropriate timing of herbicide treatments relying on these gene expression biomarkers. Several technologies will require further improvement to make this idea feasible. First, the cost of gene expression analysis for biomarker genes must be reduced. Considering the current cost of 30,000 Japanese yen for detection of approximately 180 K probes (or genes), it may be possible to reduce the cost to around 100 Japanese yen for 1000 genes. Second, we usually use liquid nitrogen for leaf sampling and storage, but this is an obstacle for wider use. Therefore, reagents for fixing RNA in rice leaves at normal temperature are needed. Finally, sampling under field conditions is laborious for farmers. As one example, automatic sampling machines, drones, and self-traveling vehicles would be useful for developing practical handling to check crops based on gene expression data. After efficient sampling, the users can send samples to the center for RNA and data analyses. The samples would be analyzed within a few days, and checkup data would be sent back to the users for diagnosis of their crops.

6.5 Potential Use of Field Transcriptome Data to Mine Genetic Resources Against Global Climate Change

Transcriptome data obtained under field conditions will be useful for breeding in the future. With global climate change, new cultivars with wide regional adaptability are required. Previously, a breeding method called shuttle breeding applied in the Green Revolution of wheat breeding was thought to be effective for developing new cultivars with wide adaptation (Hesser 2006). However, it is very laborious as the cultivars were selected in two distant areas with very different climates. Use of field transcriptome data from multiple fields with distinct climates would allow the selection of useful alleles to confer distinct responses depending on the given environments. In addition, we could evaluate which environments can give rise to distinct responses using statistical modeling. For example, a novel disease resistance gene allele that can exhibit a distinct temperature response would likely contribute to plant disease resistance within different temperature ranges. We can make use of this allele as a genetic resource to develop new cultivars with wide regional adaptability. In the process of maize domestication, ancient humans mainly would have used genetic changes in *cis*-regulatory elements of causal genes (Lemmon et al. 2012). The search for novel alleles with wide regional adaptability to improve crops would be reasonable from a historical perspective. There are three possible ways to identify candidates of such novel alleles. The first

would be the use of paired tests. As described above, we already have more than 40 paired samples in which significant detection would become very sensitive. The second would be to develop transcriptome-based models of specific traits, such as temperature responses, and compare with other models, such as LASSO models. The third is to develop environmental-driven models of gene expression. We can compare the selected values for specific parameters for each gene among tested cultivars and determine which environmental factors cause the differences of the gene expression. The third would require more than a hundred of RNA samples obtained under various cultivation conditions for each allele of tested cultivars for comparison. Known QTLs are included among candidate genes, and we would be able to identify novel useful alleles in the QTLs. If there is no available biological information on the candidate genes, the CRISPR/Cas9 method could have been applied to the candidate genes for molecular genetic analysis.

6.6 Future Crop Agronomic Performance Mediated by Field Transcriptome Data

6.6.1 Spatial and Developmental Regulation In Planta *as Barriers for Field Transcriptome Analysis*

There are critical barriers when performing transcriptome analysis for plants cultured in the laboratory and/or field. One such barrier is that the samples for transcriptome analysis are mixtures of different plant organs. Although in the case of fully developed rice leaves, the ratios of mixing of tissues, such as vascular bundle cells and mesophyll cells, do not vary markedly, so we cannot discuss tissue specificity of expressed genes in rice leaves. In addition, when performing transcriptome analysis of differentiating tissues/organs, it would be very difficult to order the samples according to developmental stages among fluctuations in gene expression according to environmental factors. Therefore, at present we can only focus on fully differentiated tissues/organs. Furthermore, the damage due to sampling may affect future samples from the same plants. We usually try to harvest samples at intervals that are as short as possible in a sampling event. These flaws must be taken into account when designing biological experiments using field transcriptome analysis.

6.6.2 Understanding of the Effects of Ambient Environmental Conditions

The final goal of field transcriptomics would be global integration between environment-driven models of gene expression and transcriptome-driven models of biological and agricultural traits (Fig. 6.4). Such integration would allow the

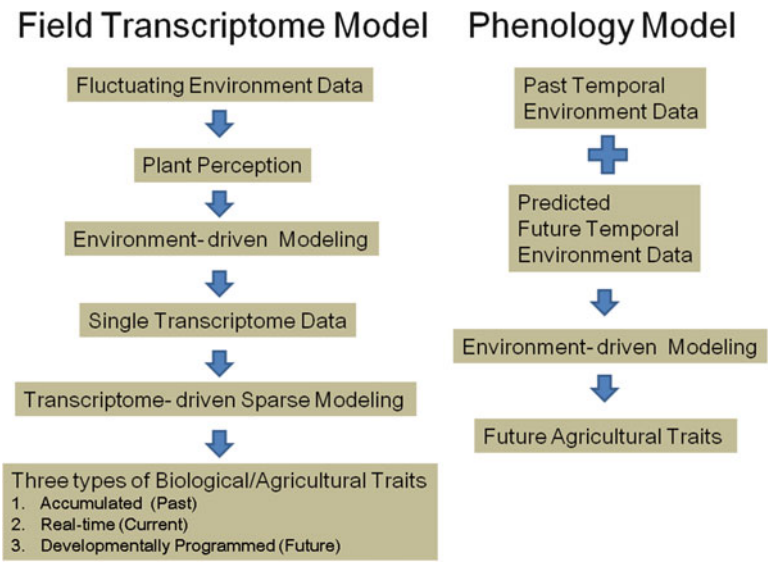


Fig. 6.4 Comparison between field transcriptome and phenology models. In the field transcriptome model, prediction of single transcriptome data is a feature in the environment-driven model. Prediction of traits by single transcriptome data is a feature in the transcriptome-driven model

biological and agricultural traits, including some future traits, such as heading date or flowering time or yield-related traits, to be predicted based on historical environmental information. These analyses would also reveal the critical relationships between given environments and traits, including distinct responses in distinct developmental stages and gate effects of timing. Such integrated views from transcriptome data would provide dynamic responses of crops under naturally fluctuating environmental conditions.

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Chapter 7
Plant Hormone Cross Talk with a Focus on Strigolactone and Its Chemical Dissection in Rice

Shinsaku Ito, Kai Jiang, and Tadao Asami

Abstract Plant hormones and several series of small molecules in plants play versatile roles in regulating plant growth and development. Since the finding of the first plant hormone auxin, the studies on both biosynthesis and signaling pathways of plant hormones have made a great progress because of the contribution of genomics and genetics. In rice, several *dwarf* (*D*) mutants that show dwarf phenotypes due to loss of functions or gain of functions in various genes have been determined to be involved in plant hormone biosynthesis or signaling pathways. Especially the studies on strigolactones (SLs) have greatly relied on *D* mutants including *D27*, *D17*, *D10*, *D14*, *D3*, and *D53*. Vice versa, SL studies deciphered how the genes regulate the phenotype and nutrition absorption in rice. In this chapter, we focus on summarizing the recent studies on the cross talk of SLs with other plant hormones to give an insight on the complexity of plant hormone signals. We also introduce and propose the combination of chemical regulators with genomics and genetics studies to drive the studies on plant forward.

Keywords Plant hormone · Cross talk · Strigolactone · Chemical regulator · Dwarf mutant

7.1 Introduction

Plant hormones regulate plant development and also play an important role in defense and immune responses. Recent intensive research on physiology and genomics of *Arabidopsis* controlled by plant hormones revealed that one of

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physiological processes such as hypocotyl elongation and so on is controlled by many plant hormones (Vanstraelen and Benková 2012). That is, as a result of plant hormone signal integration, physiological processes in plant life cycles are controlled by "one process with many hormone" mechanisms. Although almost all of the knowledge about plant hormone cross talk has been obtained by the research on *Arabidopsis*, we should go to the next stage on how to apply the results from plant hormone cross talk to crop production.

Many trials have been done to increase crop yields through chemical treatment such as pesticides, introduction of gene-modified crops, and conventional plant breeding. Among the several ways for increasing crop yields, we have been focusing our attention on chemical regulation. Chemicals are widely used for agriculture to increase yield of crops. For example, pesticides protect crops from the attack of pests that cause damage to crops, such as weeds, plant diseases, or insects. As a chemical, they can be categorized mainly to herbicide, fungicide, insecticide, and/or insect growth regulator that kill pests. That is, pesticides are negative controller of pests. While, as plant growth regulators (PGRs) are chemicals that control plant growth and are beneficial for crop production by enhancing crop quantity and quality, and by improving the postproduction quality of some plants, they are thought as positive controller of plants. In developing countries, such as China, plant growth regulator industry has seen remarkable progress and shown attractive market potential in the future (Grand View Research 2015). It is likely that PGRs will be utilized on large-volume species and cultivars.

In this chapter plant hormone cross talk in rice and its chemical regulation will be described with a focus on a new plant hormone, strigolactone.

7.2 Strigolactone Cross Talk with Other Plant Hormones in Rice

Strigolactones (SL) are a group of terpenoid lactones that cause various morphological changes significantly affecting grain yields such as tiller bud outgrowth, mesocotyl elongation, lamina joint inclination, leaf senescence, and adventitious root formation in rice (Gomez-Roldan et al. 2008; Umehara et al. 2008; Hu et al. 2014; Yamada et al. 2014; Li et al. 2014; Sun et al. 2015). Because auxin, cytokinin, gibberellin, and brassinosteroid also control similar morphological change with strigolactones, our understanding of SL signaling cross talks with these hormones continues to expand.

SL biosynthesis had been discovered by the analysis of branching mutants (Gomez-Roldan et al. 2008; Umehara et al. 2008; Lin et al. 2009): *D10* and *D17* encode carotenoid cleavage dioxygenase (CCD) 8 and 7, respectively. *D27* encodes a novel iron-containing protein. In 2011, carlactone (CL), which has a SL-like carbon skeleton, was discovered as a product of enzymatic reactions from all-trans-β-carotene by these three proteins in vitro (Alder et al. 2012). Recently,

Zhang et al. showed that two rice homologs, *Os01g0700900* and *Os01g0701400*, of *Arabidopsis MORE AXILLARY GROWTH 1* (*MAX1*), which encodes cytochrome P450 family (CYP711 subfamily), catalyze the enzymatic conversion of CL to 4-deoxyorobanchol (4DO) and of 4DO to orobanchol, respectively (Zhang et al. 2014).

SL biosynthesis is regulated by various environmental cues. For example, SL biosynthesis is activated by the upregulation of SL biosynthesis genes (*D10*, *D17*, *D27*, *Os01g0700900*, *Os01g0701500*, and *Os02g0221900*) under phosphate-deficient condition (Umehara et al. 2010), because SLs facilitate the uptake of inorganic phosphate through the symbiotic association with arbuscular mycorrhizal fungi (Akiyama et al. 2005). Recently, regulatory mechanisms of SL biosynthesis by plant hormones (auxin, cytokinin, and gibberellin) have been also discovered in rice.

7.2.1 Regulation of SL Biosynthesis by Auxin

Auxin is an essential plant hormone that regulates many aspects of plant growth and development. Auxin regulates tiller bud outgrowth via basipetal movement of auxin called as pollar auxin transport (PAT) (Arite et al. 2007). However, as auxin cannot enter the lateral bud, cytokinin and SL are known to be involved in this event as second messengers (Fig. 7.1). Thus, the research on auxin and strigolactone cross talk attracts many scientists, and the knowledge about the interaction between two hormones is now expanding. Auxin application to the leaves increased the expression levels of *D10*, *D17*, and *D27* in tiller nodes (Xu et al. 2015). In addition, NAA treatment after decapitation recovered the *D10* expression, while *D10* expression was reduced by decapitation (Zhang et al. 2010b), suggesting that PAT regulates SL biosynthesis in rice. In *Arabidopsis*, SLs control bud outgrowth via two pathways (Cheng et al. 2013). One is that SLs reduce the capacity of the PAT stream in the main stem. Another is that SLs are transported acropetally through the xylem from roots and directly inhibit bud outgrowth through the regulation of TCP transcription factor (BRC1). Similarly, GR24 treatment also reduced the expression levels of *PIN* family genes in roots and the junction of rice seedlings and changed the IAA distribution in rice (Sun et al. 2014, 2015). These results suggest that the similar capacity of the PAT stream is important to control tiller bud outgrowth in rice.

7.2.2 Cytokinin-SL Cross Talk

Cytokinin also regulates tiller bud outgrowth and is thought as one of the second messengers of auxin-mediated tiller outgrowth. Cytokinin application to the growth media reduced the expression levels of *D10*, *D17*, and *D27* in rice roots, tiller nodes, and tiller buds (Xu et al. 2015). In contrast, SL-deficient rice (*D10* RNAi

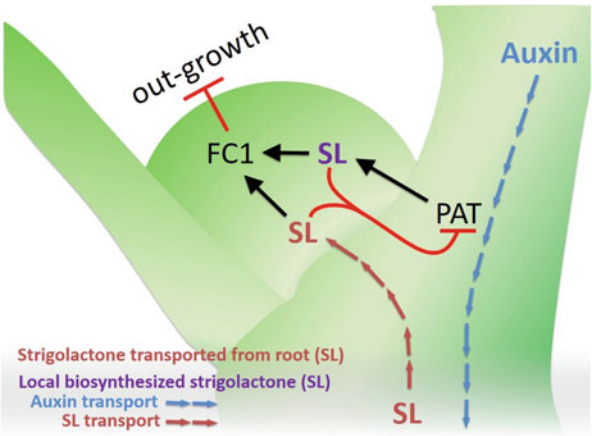

Fig. 7.1 Regulation of branching by SL and auxin. Pollar auxin transport (*PAT*) and strigolactone (*SL*) indirectly and directly regulate tiller bud outgrowth, respectively

line) showed altered endogenous levels of cytokinins: D10 prompts the increased glycosylated cytokinins and the decreased active cytokinins (Zhang et al. 2010b). In addition, cytokinin suppressed the gene expression level of *FC1*, which is a homologous gene of *Arabidopsis* TCP transcription factor, *BRC1* (Xu et al. 2015). The fact that SL treatment increases the expression level of *FC1* gene and does not affect the tiller number in *fc1* mutant indicates that *FC1* works downstream of SL and cytokinin to regulate the tiller bud outgrowth in rice (Minakuchi et al. 2010; Xu et al. 2015). These findings indicate that *FC1* gene is one of the key regulators for SL-cytokinin cross talk in tiller bud outgrowth. Recently, Hu et al. reported that SL also regulates rice mesocotyl elongation (Hu et al. 2014). In addition, co-application of cytokinin and SL induced the antagonistic effect on SL and cytokinin in mesocotyl elongation, respectively. Furthermore, *OsTCP5*, which belongs to the same group with *FC1*, was regulated by cytokinin and SL, and its expression level was negatively correlated with mesocotyl elongation. These results suggest that TCP-type transcription factors mediate cytokinin and SL-regulated phenomena and these hormones regulate their biosynthesis each other (Fig. 7.2).

7.2.3 Gibberellin-SL Cross Talk

Gibberellin (GA) is a plant hormone that is an important growth regulator in higher plants. Plants defective in GA biosynthesis and signaling show characteristic phenotypes, including dwarfism, male sterility, reduced seed production, dark green leaves, and promotion of tiller outgrowth (Lo et al. 2008; Yamaguchi 2008). The activities of GA depend on *GID1*, *GID2*, and *SLR1*, which encode GA receptor, F-box protein, and DELLA protein, respectively. Perception of GA to GID1 leads to the 26S proteasome-dependent degradation of SLR1 (Ueguchi-

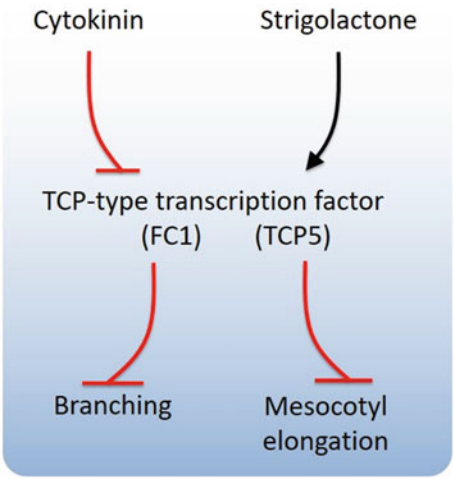

Fig. 7.2 Illustration of SL-CK cross talk. TCP-type transcription factors mediate SL- and CK-regulated phenomena

Tanaka et al. 2007). SL also shows similar feature in perception. SL binding to SL receptor, called as D14, triggers the interaction with D53 and D3 proteins, which are the repressor protein and F-box protein in SL signaling, respectively. After the formation of ternary complex, D53 protein is ubiquitinated and degraded (Jiang et al. 2013; Zhou et al. 2013). In addition, functional overlap between SL and GA, such as regulation of tiller bud growth, suggests the presence of SL-GA cross talk. Actually, Nakamura et al. found the SL-dependent molecular interaction between D14 and SLR1 by yeast two-hybrid and bimolecular fluorescence complementation analyses (Nakamura et al. 2013). Competition assay using the yeast three-hybrid system, which uses D14 as the bait, SLR1 as the prey, and GID1 as a D14 competitor, revealed that the formation of SL-dependent D14-SLR1 interaction is inhibited by the GA-dependent GID1-SLR1 formation, and the D14-interacting domain overlaps with the GID1-interacting domain in SLR1 (Nakamura et al. 2013). These results indicate the existence of novel SL-GA cross talk in rice. However, contradicting results have been reported that SL independently acts internode elongation of GA in the pea (de Saint Germain et al. 2013). Recently, Ito et al. screened for SL biosynthesis inhibitor and found GA as a novel SL biosynthesis regulator (Ito et al. 2017). The regulatory mechanism of SL biosynthesis by GA was dependent on the GID-DELLA signaling pathway. In addition, the expression levels of SL biosynthesis genes (*D27*, *D17*, *D10*, *Os01g0700900*, and *Os01g0701400*) were downregulated by gibberellin signaling. These results indicate the existence of physiological cross talk between SL and GA signaling in rice (Fig. 7.3). However, key regulatory gene(s) were not identified in SL-GA cross talk. Treatment of protein synthesis inhibitor, cycloheximide, canceled the GA-dependent regulation of SL biosynthesis, suggesting that the regulation of SL levels by GA signaling is attributable to the altered expression of SL biosynthesis genes via de novo protein synthesis such as unknown transcription factor(s). These factors may control SL-GA cross talk. Until now, though the molecular

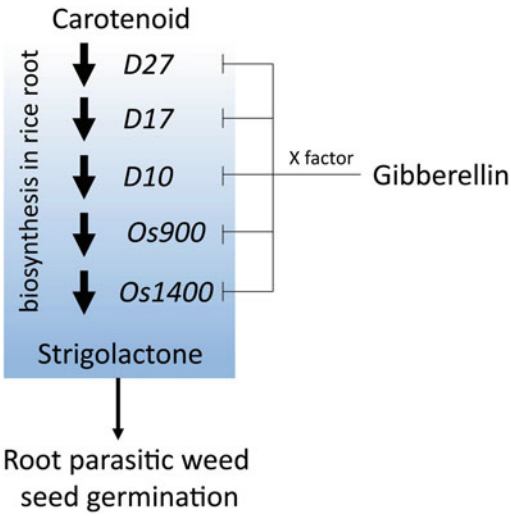

Fig. 7.3 Illustration of SL-GA cross talk. Gibberellin shows suppressive effects on strigolactone biosynthesis genes in rice root through de novo protein synthesis of unknown X factor(s). Thus treatment with gibberellin decreases the endogenous and extruded SL, which is expected to prevent root parasitic weed seed germination

mechanisms are not uncovered, further studies will lead us to find unknown factors between SL-GA cross talks. Here we showed a putative model how GA suppresses branching (Fig. 7.4) (see also Chap. 9).

SL is also a germination stimulant for root parasitic weeds, *Orobanche* and *Striga* spp., which are one of the most harmful pests. In Africa, it has been reported that approximately 300 million people are affected economically by *Striga* spp. with estimated losses of US$ 7 billion (Parker 2009). As SL-deficient or GA-treated rice was resistance to *Striga* infection (Umehara et al. 2008; Ito et al. 2017), modulation of GA signaling in rice roots becomes a useful method to control the damage by root parasitic weeds. In fact, GA treatments in rice roots reduce the levels of SLs in roots and root exudates at concentrations as low as 10 nM (Ito et al. 2017). In addition, GA treatment at 10 nM did not affect the rice tiller number. These results strongly suggest its usefulness as the method for controlling root parasitic weeds (Fig. 7.3).

7.2.4 Brassinosteroid-SL Cross Talk

Brassinosteroids (BRs) are a group of naturally occurring steroidal plant hormones that regulate many stages of the plant life cycle (Fujioka and Yokota 2003; Sasse 2003). BRs have various physiological effects that influence cellular responses such as cell division, cell elongation, xylem differentiation, hypocotyl elongation (Müssig 2005), leaf expansion (Asahina et al. 2014), pollen germination (Singh and Shono 2005), and those processes related to the regulation of gene expression (Clouse and Sasse 1998). BRs are also involved in both biotic and abiotic

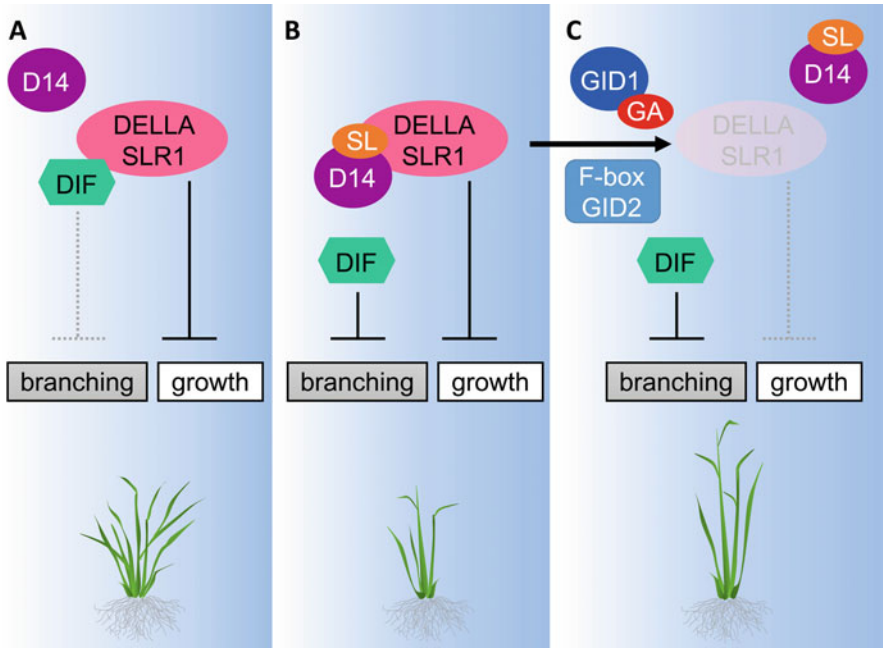

Fig. 7.4 Model on how GA reduces branching in rice: (**a**) DELLA-interacting factor (*DIF*) cannot function due to its binding to DELLA, which attenuates DIF's suppressive effect on branching. (**b**) High concentration of SL induces the formation of D14-SL-DELLA complex, and the released DIF can function to inhibit branching. (**c**) High concentration of GA induces the degradation of DELLA and consequently releases DIF. Eventually, GA promotes plant growth and suppresses branching

resistances of plants, e.g., disease, cold stress, temperature stress, and salt stress (Clouse and Sasse 1998; Krishna 2003).

In *Arabidopsis*, the GSK3/SHAGGY-like kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2) plays a critical role in the brassinosteroid (BR) signaling pathway by negatively regulating the activities of bri1-EMS-SUPPRESSOR1/BRASSINAZOLERESISTANT1 (BES1/BRZ1) family transcription factors that regulate the expression of downstream BR-responsive genes (Wang et al. 2002; Yin et al. 2002). When one of the orthologs of *BIN2* in rice, *GSK3/SHAGGY-like kinase* (*GSK2*), is overexpressed, rice showed typical BR loss-of-function phenotypes, while suppression of *GSK2* in rice resulted in enhanced BR signaling phenotypes. Suppression of *DWARF AND LOW-TILLERING* (*DLT*) in rice can enhance the phenotypes of BR receptor mutant *d61-1*, and overexpression of *DLT* clearly suppressed the BR loss-of-function phenotypes of both *d61-1*. This result suggests that DLT should function downstream of GSK2 to modulate BR responses. As rice tillering is strongly connected with the function of SL, this result may suggest the existence of cross talk between BRs and strigolactone (Tong et al. 2012). Then, how can BRs cross talk with SL? The direct evidence of the cross talk

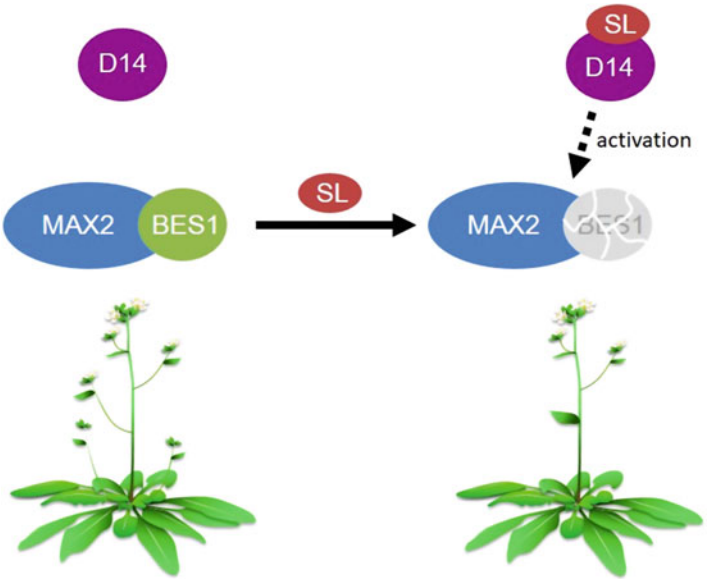

Fig. 7.5 Cross talk between SL and BR in regulating but outgrowth that is mediated by MAX2 and BES1 (Adapted from Wang et al. 2013). Strigolactone (*SL*) activates the degradation of BES1, which functions at the downstream of MAX2 in regulating branching

between BRs and SL comes from the protein-protein interaction in *Arabidopsis* (Wang et al. 2013).

MAX2, a subunit of an SCF E3 ligase, is a positive regulator inhibiting shoot branching and is a key signaling component both for SL and karrikin in *Arabidopsis*. Wang et al. demonstrated the genetic and biochemical evidence that BES1 interacts with MAX2 and acts as its substrate to regulate SL-responsive gene expression (Wang et al. 2013) and that a noncanonical SL receptor, AtD14, can promote BES1 degradation. Downregulation of *BES1* and its homologs strongly suppressed the branching phenotype of *max2-1* mutant. Above results clearly demonstrate that the SL and BR signaling pathways distinctly regulate the same transcription factor, BES1, to control specific developmental processes (Fig. 7.5). This discovery provides insight into a significant mechanism by which BR and SL signaling pathways share common transcription factor, BES1. That is, BES1 is a key component for BR-SL cross talk. BZR1 is also an interactor and degradation target of MAX2, but it may not be a major factor to inhibit the SL-mediated shoot branching because unlike *bes1D* plants, the light-grown *bzr1-1D* plants have slightly dark green leaves, shorter hypocotyls, and shorter petioles than the wild type (Wang et al. 2002) and also normal branching. Therefore, BZR1 may be involved in other MAX2-mediated developmental processes, which need to be explored. Recent studies showed that D53 acts as a substrate of D3 to regulate tiller number in rice (Jiang et al. 2013; Zhou et al. 2013). Wang et al. demonstrated that BES1, a key transcription factor in brassinosteroid (BR) signaling pathway,

functions as a substrate of MAX2, an ortholog of the rice D3, for degradation to regulate shoot branching in *Arabidopsis* (Wang et al. 2013). Both *d53*, a dominant mutant of *D53*, and *bes1D*, a *BES1* gain-of-function mutant, have increased tiller number or enhanced shoot branching in rice and *Arabidopsis*, respectively.

In addition to the enhanced shoot branching, SL biosynthesis and signaling mutants in rice and *Arabidopsis* also exhibit many other phenotypes at different stages, such as reduced plant height (Ishikawa et al. 2005) and promoted mesocotyl elongation (Hu et al. 2010) in rice, increased lateral root number (Kapulnik et al. 2011) and reduced secondary growth (Agusti et al. 2011) in *Arabidopsis*, and decreased root length (Ruyter-Spira et al. 2011; Arite et al. 2012) in both *Arabidopsis* and rice. These observations indicate that SLs play diverse roles in the processes of plant growth and development. Recently Li et al. reported that other than increase in tiller number, SL-related dwarf mutants with enhanced tillering have other altered aspects of rice architecture, that is, leaf erectness (Li et al. 2014). After growing for 12 days, SL-related *d* mutants exhibited increased lamina joint angle (defined as the angle between the first leaf blade from bottom and the upper vertical culm). The lamina joint angles of the SL biosynthetic mutants, *d17*, d10, and *d27*, and the SL signaling mutants, *d3* and *d14*, ranged from 54 to 90°, while the leaf angle of wild type (cv. Shiokari) was about 24°. Interestingly, the lamina joint angle of *d3* mutant was about 90°, which was dramatically larger than that of other SL-related *d* mutants (Li et al. 2014). It is well known that BRs can enhance lamina joint inclination in rice (Wada et al. 1981). Although the cellular mechanism of the increased lamina joint inclination in the SL-related mutants is likely different from that in the typical BR signaling mutants, it is well known that BRs can enhance lamina joint inclination in rice (Wada et al. 1981). BRs induce cell elongation in the adaxial side of the lamina joints, resulting in leaf bending (Zhang et al. 2010a). However, there is no significantly enhanced cell elongation in the adaxial side of the lamina joints in the *d10* and *d3* mutants. Therefore, protein-protein interaction between BES1 and MAX2 is likely not involved in this lamina inclination; it is of great interest to further understand the underlying molecular mechanism of how SLs participate in regulating leaf erectness and plant architecture in rice at different developmental stages.

7.3 Chemical Dissection of Strigolactone Signals from Other Plant Hormones

Chemicals that regulate functions of SLs can be widely used for agricultural applications. Also in plant sciences, the importance of the chemicals that control SL function has recently been increasing. Firstly, the advantage of using bioactive chemicals for analyzing the roles of SL in plants, rather than SL-deficient mutants, is that it can be applied regardless of the plant species. Phenotypic changes induced by chemical treatment can reveal physiological functions associated with target

proteins. Secondly, genetic redundancy does not significantly influence the effects of inhibitors. Plants treated with an antagonist or a biosynthesis inhibitor show an almost equal phenotype like as the multiple mutants when the target protein is redundant. Finally, chemicals easily regulate the functions of target protein temporally (Kitahata and Asami 2011; Nakamura et al. 2013). That is, utilization of biosynthesis inhibitors or receptor inhibitors is a useful alternative way to dissect biological processes instead of mutations (Blackwell and Zhao 2003). In this context, we developed several chemical regulators for SL functions, such as biosynthesis inhibitors and receptor inhibitors. As we have already reviewed SL function regulators reported until 2014 (Nakamura and Asami 2014), here we briefly summarized newly released chemical regulators except agonists for SLs.

As described above in this report, gibberellin can suppress SL biosynthesis and has a potential for reducing damage by root parasitic weeds. However, commercially available gibberellin is very expensive, and therefore it is not practical to use gibberellin for controlling *Striga* germination. Alternative way is to apply gibberellin agonists to the field in sub-Saharan Africa. AC94377, an N-substituted phthalimide, is a commercial chemical that mimics the growth-regulating activity of GAs in various plants (Fig. 7.6). Recently we confirmed that AC94377 displays GA-like activities in *Arabidopsis* and demonstrated that AC94377 binds to the *Arabidopsis* GID1 receptor (AtGID1), forms the AtGID1-AC94377-DELLA complex, and induces the degradation of DELLA protein (Jiang et al. 2017a). Our results also indicated that AC94377 is selective for a specific subtype among three AtGID1s and that the selectivity of AC94377 is attributable to a single residue at the entrance to the hydrophobic pocket of GID1. To discover novel GA mimic, we performed a chemical library screening and identified a chemical, named 67D, as a stimulator of seed germination that was suppressed by paclobutrazol (PAC), a GA biosynthesis inhibitor (Fig. 7.6) (Jiang et al. 2017b). In vitro binding assays indicated that 67D binds to the GID1 receptor. Further studies on the structure-activity relationship identified a new chemical that strongly promoted seed germination suppressed by PAC. This new chemical was further confirmed to promote the degradation of RGA (for repressor of ga1–3), a DELLA protein, and to suppress the expression levels of *GA3ox1* in the same manner as GA does. AC94377, 67D, and its analogs are agonists of GID1 and are expected to be utilized in agriculture and basic research as an alternative to GA.

Antagonists of plant hormone receptors are expected to give a direct control on hormone signals. For example, auxinole, coronatine-O-methyloxime (COR-MO), and a 3′-alkylsulfanyl ABA with six-carbon alkyl substitution were reported to be antagonists of auxin receptor TIR1, jasmonic acid receptor COI1, and ABA receptor PYL, respectively (Hayashi et al. 2008, 2012; Takeuchi et al. 2014; Monte et al. 2014). These chemicals block the interactions of target receptors with their partner proteins, AUX/IAA for TIR1, JAZ for COI1, and PP2C for PYL, and thus suppress the plant hormone signal transduction. Although the agonists of SL receptor have been largely reported, the antagonist is still rare. To develop antagonist for controlling SL signal, we performed virtual screening in silico by analyzing a pharmacophore model based on the published structural information of SL receptor

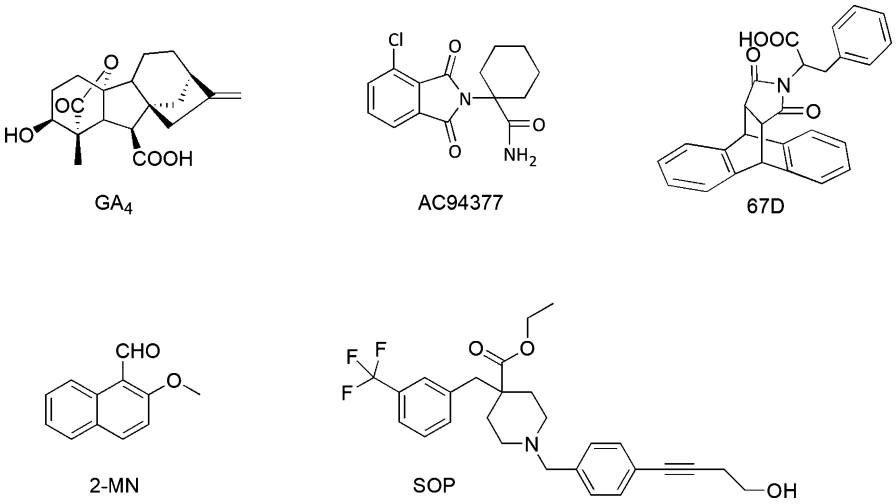

Fig. 7.6 Chemical structures of agonists of GID1 and antagonists of SL receptor

D14 (Mashita et al. 2016). A chemical, 2-methoxy-1-naphthaldehyde (2-MN) (Fig. 7.6), was finally confirmed to show antagonistic activities in in vitro and in vivo experiments, such as that 2-MN inhibits SL-induced D14-SLR1 and D14-D53 interaction in yeast two-hybrid assay and restores SL-suppressed second tiller growth in rice. 2-MN also showed inhibitory effect on the SL-induced *Striga* seed germination, albeit this activity was not as strong as its effects in restoring second tiller growth in rice. Another recent chemical screen performed by Holbrook-Smith et al. identified a compound, soporidine (SOP) (Fig. 7.6), to show antagonistic effects against GR24 in inducing *Striga* seed germination (Holbrook-Smith et al. 2016). Unlike 2-MN, no obvious phenotypic change in rice was observed with treatment of SOP.

Up to now, remarkable achievements of studies on SL have portrait its biosynthesis and signaling pathways. The rice *dwarf* (*d*) mutants, including *d10*, *d17*, and *d27* for SL biosynthesis studies and *d14*, *d3*, and *d53* for signaling studies, have made great contributions to these achievements. However, there are still several open questions to be answered. (1) Although the crystallization of AtD14-CLIM-D3 complex was reported by Yao et al. (2016), the process that how D14 recruits D53/SMAX1/SMXLs and induces its degradation is still to be further clarified. (2) The SL-related signal factors downstream of D53 and their mode of action are still largely unknown. (3) The details of cross talk between SL and other hormones in regulating plant growth and development are worth to be investigated. Chemical biology is a powerful tool to study signaling pathway; thus development of SL agonist and antagonist and application of other plant hormone regulators are expected to facilitate the studies on SL signal in combination with genomic and genetic studies.

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Chapter 8
Rice Plant Architecture: Molecular Basis and Application in Breeding

Shiwei Bai, Steven M. Smith, and Jiayang Li

Abstract Shoot architecture in rice is determined by the number of leaves, stems, and panicles and by their size, shape, and position on the plant. These factors determine the effectiveness of light interception, the degree of competition between neighboring plants, and ultimately the number and mass of grains produced. Plant hormones including auxin, cytokinins, gibberellins, strigolactones, and brassinosteroids play key roles in regulating shoot development and architecture. The *SEMI-DWARF1* (*SD1*) gene has contributed greatly to rice yields by redirecting resources from elongation growth to panicle development, providing resistance to lodging and increased harvest index. The mechanism of control of tillering by strigolactone signaling has been determined in recent years providing valuable information to help understand the timing and number of tillers produced. Genes that have been selected for increased yield have now been identified at the molecular level such as *IDEAL PLANT ARCHITECTURE1* (*IPA1*), *Grain size 3* (*GS3*), and *GRAIN NUMBER*, *PLANT HEIGHT*, *AND HEADING DATE 7* (*GHD7*). The function of these genes in controlling gene transcription and shoot development is helping us to understand the molecular basis of plant architecture.

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The future offers great potential for the rational design of plant architecture using molecular breeding techniques.

Keywords Tiller number · Panicle development · Leaf development · Dwarfing genes · Ideal plant architecture · Phyllotaxy · Auxin · Cytokinins · Gibberellins · Strigolactones · Brassinosteroids · Molecular breeding · Rational design

8.1 Architecture of the Rice Plant

8.1.1 Plant Architecture Is Dynamic

In crop plants, differences in architecture and changes in architecture can have a profound impact on crop performance and productivity. For example, the architecture of the leaves determines how much light is intercepted, while stem branching not only determines leaf number but also the number of flowers and seeds produced (Wang and Li 2008).

The architecture of a plant is not simply a description of its shape and structure, but it also includes the "design and construction" process, otherwise known as growth and development. Furthermore, the design enables the structure to fulfill a set of functions or to perform in a particular way. Since the functions change during the lifetime of a plant, and since the circumstances in which the plant operates also change with time, plant architecture must be dynamic.

We should not only understand how the architecture is created but also how it functions, so that we can design or select plants that perform better and have higher yield potential. Changing the architecture is achieved by changing the developmental processes since the architecture is a product of development (Fig. 8.1).

8.1.2 Phyllotaxy and Phytomers

The structural plan is determined by meristems. The shoot apical meristem (SAM) gives rise to leaf primordia from which leaves emerge in a defined pattern, which in rice and other grasses is distichous. This means that leaves arise alternately on opposite sides of the stem. In contrast in *Arabidopsis* leaves arise in a spiral, with 137.5° between each leaf. Such patterns of leaf development, or phyllotaxy, are genetically determined and brought about by the spatial distribution of auxin, cytokinin, and peptides influencing the expression of key developmental genes (Smith et al. 2017). In rice, the *DECUSSATE* gene acts in cytokinin signaling in the SAM, and mutation results in decussate (paired, opposite) phyllotaxy instead of distichous (Itoh et al. 2012). Similarly in maize, *abphyl1* mutant plants also initiate leaves in a decussate pattern, again through changes to cytokinin signaling (Giulini et al. 2004).

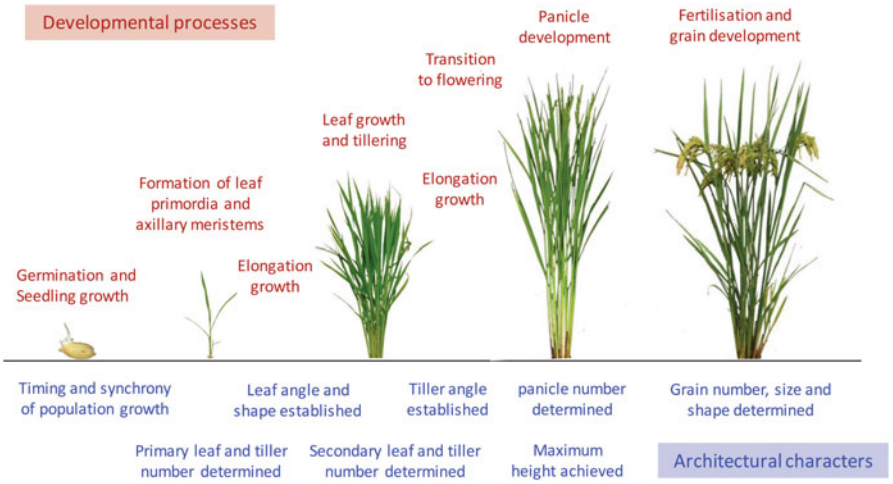

Fig. 8.1 Development of rice plant architecture. Many of the developmental process (shown in red) determine the architectural characters (blue) of the mature plant

Within the axil of each leaf, a secondary or axillary meristem can arise, either during the process of leaf initiation in the SAM or later as the leaf develops. The axillary meristem can give rise to a secondary stem or tiller, in a two-step process. First, the meristem develops into a bud which is initially dormant but can later be activated to grow into a secondary shoot or tiller (see Sect. 8.4).

The number of tillers is defined initially by the number of leaves on the primary stem because there is only one axillary meristem per leaf. However the primary tillers produce new leaves which can produce secondary tillers from their leaf axils, which can in turn produce tertiary tillers. While multiple tillers can therefore be produced, the number is always fewer than the number of leaves (see Sect. 8.4).

The repeating unit comprising a node, internode, leaf, and axillary bud is known as a phytomer. New phytomers are added by the formation of new leaf primordial at the apical meristem and elongation of the stem. Growth is therefore achieved by increasing both the number of phytomers and their subsequent size. The modular phytomer system also allows changes in shoot architecture as a result of the outgrowth of lateral buds, producing additional tillers and subsequently additional panicles.

8.1.3 Phyllochron and Phenology

The rate at which leaf primordia are produced is known as the plastochron, while the rate at which new leaves appear is known as the phyllochron. These are very important factors in the establishment of shoot architecture. If the phyllochron is short, tillers can be produced within a shorter time period and hence can be much

more uniform in size, and panicles can develop synchronously. Such factors are very important for yield and uniformity in cereals (McMaster 2005).

Rice is a short day plant and flowers when day lengths are less than about 12 h (Yano et al. 2001). The transition from vegetative to reproductive growth plays a major role in determining plant architecture because the flowering process triggers an end to tiller formation and signals the development of panicles. The timing is vital in crops to ensure that the optimal vegetative structure has been created to support optimum grain production (Yano et al. 2001).

8.2 Past Progress in Breeding for Architecture and Yield

The discovery and introduction of semidwarf genes had a tremendous impact on rice production. The semidwarf *sd1* mutant was discovered in the 1950s in China from which the high-yielding variety Guang-Chang-Ai was developed. In the International Rice Research Institute (IRRI) in the 1960s breeders developed a high-yielding *indica* rice variety, IR8, which contains a semidwarf gene introduced from Dee-geo-woo-gen (IRRI 1967; Peng et al. 1994; Ashikari et al. 2005). Such semidwarfing genes reduced susceptibility to lodging and allowed greater resource allocation to panicles and seeds.

A rice breeding program was started in 1982 in Japan aimed at producing "super high yield" (Qian et al. 2016) which focused on large-panicle traits. However, somewhat less attention was paid to other traits including grain quality and fertilizer use, and the increases in yield achieved were relatively modest. In 1989 IRRI launched a breeding program to create a "new plant type" (NPT) also known as "Super Rice" (Peng et al. 2008; Xue and Qian 2007; Khush 2001). The original NPT was proposed to have only 5–6 tillers per plant, 200–250 grains per panicle, plant heights of 90–100 cm, and 100–130 days growth duration (Peng et al. 1994). This was later adjusted to increase plant height and panicle numbers and reduce the number of grains per panicle and later revised again to aim for 150 spikelets per panicle and approximately ten panicles.

Similarly in China a "Super Rice" program was launched in 1996 aimed at using both heterosis and an "ideal plant type" strategy to create new varieties with increases in yield (Chen et al. 2007). The standards for the "ideal plant type" were different for each of the major rice production areas in China. Three models proposed were the erect panicle type, the early vigorous growth and heavy panicle type, and the type with long erect leaves and delayed senescence. Many new hybrids and inbred rice varieties were released, and some achieved good yield increases (Qian et al. 2016). Thus significant advances were achieved in these programs, but the approaches reflected the lack of knowledge of how to modify architecture to increase yield.

In the twenty-first century, the determination of the rice genome sequence and establishment of detailed genetic maps with a high density of molecular markers have enabled many genes to be identified that control shoot architecture (Qian et al.

2016). Such genes regulate leaf shape and position, plant height, tiller number, tiller angle, number of tillers, panicle number, and panicle angle and shape.

8.3 Plant Height

8.3.1 The Benefit of Semidwarfing Genes

Plant height is mainly determined by stem elongation and has been a decisive factor contributing to crop architecture and yield (Wang and Li 2008). The identification of semidwarf mutants in cereals led to the development of new varieties that could benefit greatly from nitrogen fertilizers without growing too tall and suffering from lodging due to wind and rain. These semidwarf varieties invested a greater proportion of their resources into grain rather than shoot biomass, resulting in an increased harvest index and large increases in yield. Wheat, barley, rice, and other grain cereals were improved by such dwarfing genes. These new varieties underpinned the "Green Revolution" of the last century (Qian et al. 2016).

8.3.2 Identity of Dwarfing Genes

About 30 genes controlling plant height have been identified through reverse genetics and map-based cloning. Most of them function in the biosynthesis or signal transduction of gibberellins (GAs), brassinosteroids (BRs), and strigolactones (SLs). The most commonly used and most important gene in breeding is the *semi-dwarf 1* (*sd1*) gene which encodes a gibberellin 20-oxidase. This enzyme oxidizes the inactive GA_{53} to the inactive GA_{20} at the carbon-20 position. GA_{20} is an immediate precursor to the active GA_1. The stimulation of rice stem growth by gibberellins is well known from the original discovery that the fungus *Gibberella fujikuroi* produces gibberellin and causes the "foolish seedling" disease "bakanae" in rice. Therefore the phenotype of *sd1* is explained by a deficiency in active GA_1 in stems.

Most of the dwarf mutants identified in rice resulting from mutations in GA biosynthetic genes are associated with severe dwarfism, sterility, or abnormal grain development so are not used in breeding (Spielmeyer et al. 2002). The success of *sd1* is probably be due to the moderate dwarfing effects on stem growth and panicle development which might be explained by the timing and distribution of its expression.

In wheat, *Reduced height* (*Rht*) genes are used to create semidwarfs. The *Rht* genes encode DELLA proteins which are downstream targets of GA signaling, and the loss-of-function mutants are unresponsive to GA. In rice, however, the equivalent loss-of-function mutant known as *slender1* (*slr1*) is not useful in breeding because its development is severely compromised. Similarly, the rice *gibberellin-*

insensitive1 (*gid1*) mutant which lacks a functional GA receptor and the *gid2* mutant which lacks a functional GA-signaling F-box protein are not useful in breeding.

A major factor in determining plant height is GA action (Sakamoto and Matsuoka 2004). In recent years, a number of genes involved in the GA biosynthetic pathway have been cloned, such as *ent-Kaurene Synthase 1* (*KS1*), *DWARF 18* (*D18*), *DWARF 35* (*D35*), *SEMI-DWARF 1* (*SD1*), *Gibberellin 2-oxidases* (*GA2ox*), and *ELONGATED UPPERMOST INTERNODE 1* (*EUI1*). The *KS1* gene encodes an *ent*-kaurene synthase (Margis-Pinheiro et al. 2005). The *D35* gene encodes *ent*-kaurene oxidase (KO) (Itoh et al. 2004). The rice "Green Revolution" gene *SD1* encodes GA20 oxidase (GA20ox) (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). The *D18* gene encodes a GA 3β-hydroxylase 2 (GA3ox2) (Itoh et al. 2001). The *GA2ox* gene encodes the enzyme GA2-oxidase (Lo et al. 2008). The *EUI1* gene encodes a cytochrome P450 monooxygenase (Zhu et al. 2006; Luo et al. 2006). KS1 and KO catalyze early steps in the GA biosynthesis pathway. GA20ox and GA3ox2 are responsible for the conversion of GA from an inactive to a bioactive form, whereas GA2ox and EUI1 catalyze conversion of bioactive GAs or their immediate precursors to inactive forms. Taken together, rice plant height can be regulated through the levels of active GAs. Besides the GA biosynthetic pathway, many genes involved in the GA-signaling pathway also affect the plant height. The *GID1* gene encodes a soluble GA receptor mediating GA signaling (Ueguchi-Tanaka et al. 2005, 2007). Downstream of GID1, the *D1* gene encodes the α-subunit of GTP-binding protein (Ashikari et al. 1999; Ueguchi-Tanaka et al. 2000; Fujisawa et al. 1999). The *SLR1* gene encodes a rice DELLA protein, which is a target of GA signaling in rice (Ikeda et al. 2001; Hirano et al. 2010), while *GID2* encodes a putative F-box subunit of a Skp1-cullin-F-box (SCF) E3 ubiquitin ligase (Gomi et al. 2004; Sasaki et al. 2003). Upon the binding of bioactive GA, the conformation of GID1 is changed, which in turn promotes the formation of the GA-GID1-DELLA complex. The formation of this complex promotes the ubiquitination of SLR1, which is then degraded by the 26S proteasome (Hirano et al. 2010). In rice, GA signaling plays an important role in regulating plant height and is very important in plant breeding (see also Chap. 9).

Signaling by BR also plays a role in regulating plant height. Mutations in key genes of the BR-biosynthetic pathway also cause dwarf phenotypes, such as *BR-DEFICIENT DWARF 1* (*BRD1*), *BRD2*, *D2*, *D4*, and *D11*. The *BRD1* gene encodes brassinosteroid C-6 oxidase (BR6ox), which belongs to a member of the cytochrome P450 family. BR6ox is a key enzyme of the BR biosynthetic pathway (Hong et al. 2002). *BRD2* encodes the rice homolog of *Arabidopsis* DIMINUTO or DWARF1 protein (Hong et al. 2005), *D2* encodes a cytochrome P450 (CYP90D) (Hong et al. 2003), *D4* also encodes a cytochrome P450 (CYP90B2) (Sakamoto et al. 2006), and *D11* encodes a novel cytochrome P450 (CYP724B1) (Tanabe et al. 2005). The loss of function of these genes leads to a dwarf phenotype in rice.

Genes involved in BR signal transduction also influence plant height, such as *D1*, *D61*, *BRI1-Associated Kinase 1* (*BAK1*), *BRASSINAZOLE-RESISTANT 1* (*BZR1*), *DWARF AND LOW-TILLERING* (*DLT*), and *GSK3/SHAGGY-like Kinase 2* (*GSK2*). Besides being insensitive to GAs, the *dwarf1* (*d1*) mutant is also insensitive to BRs, and it may act as a "hub gene" in the regulation of GA and BR pathways (Wang et al. 2006). A *D1* genetic interactor *Taihu Dwarf1* (*TUD1*) encodes a functional U-box E3 ubiquitin ligase. D1 protein directly interacts with TUD1, and they act together to mediate BR signaling (Hu et al. 2013). The *dwarf61 (d61)* mutant is the first identified rice BR-insensitive mutant. The *D61* gene also known as *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) encodes a receptor-like kinase (Yamamuro et al. 2000; Nakamura et al. 2006). BAK1 acts as a BR co-receptor with BRI1 mediating BR signaling (Li et al. 2009a). The use of RNA interference (RNAi) suppression *BZR1* expression results in dwarfism, erect leaves, and reduced BR sensitivity (Bai et al. 2007). The *DWARF AND LOW-TILLERING* (*DLT*) gene encodes a member of the plant-specific GRAS protein family (Tong et al. 2009). The *dlt* mutant plants show dwarf and low-tillering phenotype, reduced BR sensitivity, and induced expression of BR-biosynthesis genes. A recent study shows that DLT is the direct downstream target of GSK2, which can interact with and phosphorylate DLT (Tong et al. 2012) (see also Chap. 7).

Plant height is also influenced by SL signaling. Both SL biosynthesis and SL signaling mutants have dwarf phenotypes, but these mutants also exhibit high tillering. It is commonly the case that high-tillering mutants exhibit dwarf phenotypes, and although the mechanism is not understood, it might simply be due to competition between multiple tillers. The control of tillering is of vital importance in rice breeding, and much attention has been paid to the molecular mechanisms controlling rice tiller number and tiller growth.

8.4 Control of Tillering in Rice

8.4.1 Different Steps in Tillering

Rice tillering occurs in a two-stage process. The first stage is the formation of an axillary bud at each leaf axil which occurs during the development of leaves from the SAM. The growth of such axillary buds is usually arrested until changes in hormones and nutrients trigger their outgrowth, leading to the formation of a tiller. Therefore tiller number is determined firstly by the number of leaves and number of functional axillary buds that are produced and secondly by the control of their outgrowth. The other vital element that influences plant productivity is the timing of outgrowth since tillers that grow late in development will likely be small and unproductive.

8.4.2 Genes Regulating the Formation of Axillary Buds

The first identified key regulator controlling rice tiller number is *MONOCULM 1* (*MOC1*), which is required for the initiation of rice axillary buds. *MOC1* encodes a plant-specific GRAS family transcription factor. The *moc1* mutant shows a single culm phenotype owing to a defect in tiller bud formation (Li et al. 2003). The *MOC3* gene encodes a nuclear-localized transcriptional repressor of the *WOX* gene family and is also required for the formation of axillary buds (Lu et al. 2015; Tanaka et al. 2015). Other genes involved in the formation of axillary meristems throughout the development of rice plants are *LAX PANICLE 1* (*LAX1)* and *LAX2*. The *LAX1* gene encodes a basic helix-loop-helix (bHLH) transcription factor, and mutants in this affect axillary meristem (AM) development (Oikawa and Kyozuka 2009). The *LAX2* gene encodes a novel nuclear protein that contains a plant-specific conserved domain and physically interacts with LAX1 (Tabuchi et al. 2011). LAX1 and LAX2 have a unique role in AM maintenance. Double mutant analyses revealed that *LAX1*, *LAX2*, and *MOC1* have partially redundant functions in AM establishment and maintenance (Tabuchi et al. 2011). *MOC3* may work downstream of *MOC1* and *LAX1* or through an independent pathway (Tanaka et al. 2015).

For the formation of axillary buds, only a few genes have been identified and cloned, and their mechanism of action is unknown. Many BR-related dwarf mutants do have more tillers, but it is not clear if this is due to an effect on the number of axillary meristems or their outgrowth (Tong et al. 2009). Mutants in SL biosynthesis and signaling have multiple tillers due to increased numbers that grow.

8.4.3 Gene Regulating Axillary Bud Outgrowth

Strigolactones (SLs) have been defined as a new group of plant hormones which inhibit bud outgrowth and hence shoot branching in higher plants. Studies on high-tillering dwarf (HTD) and dwarf (D) mutant plants in rice enabled the identification of key proteins involved in SL biosynthesis and signaling, such as *D3*, *D10*, *D14*, *D17*, *D27*, and *D53*. Other genes identified include two that encode cytochrome P450 enzymes recognized due to their homology with *MORE AXILLARY GROWTH1* (*MAX1*) from *Arabidopsis* (Stirnberg et al. 2002). The rice *OsMAX1* genes are denoted *Os900* and *Os1400*.

Biosynthesis of SL starts with the conversion of all-*trans*-β-carotene into carlactone (CL). *D27* encodes a β-carotene isomerase which converts all-*trans*-β-carotene into 9-*cis*-β-carotene (Lin et al. 2009; Alder et al. 2012). *D17* encodes carotenoid cleavage dioxygenase (CCD) family protein OsCCD7 which

catalyzes the 9,10 cleavage of 9-*cis*-β-carotene to produce 9-*cis*-β-apo-10-′-carotenal (Alder et al. 2012; Zou et al. 2006). *D10* encodes another CCD family protein OsCCD8 which catalyzes a complex oxygenation and rearrangement of 9-*cis*-β-*apo*-10′-carotenal, generating carlactone, an intermediate leading to the production of SLs (Alder et al. 2012; Arite et al. 2007). The *Os900* gene encodes a carlactone oxidase (CO) and catalyzes the oxidation of carlactone to form *ent*-2′-*epi*-5-deoxystrigol which is now known as 4-deoxyorobanchol (4DO). The Os1400 protein catalyzes the hydroxylation of 4DO to form orobanchol (Zhang et al. 2014b; Cardoso et al. 2014) (see also Chap. 7).

The D3, D14, and D53 proteins are involved in the SL signaling pathway. The *D3* gene encodes an F-box protein with Leu-rich repeat, which plays a role in SCF complex formation (Ishikawa et al. 2005). The D14 protein is a member of the *α*/*β*-fold hydrolase superfamily commonly containing a catalytic triad, Ser, His, and Asp (Arite et al. 2009; Zhao et al. 2013; Kagiyama et al. 2013). D53 is a class I Clp ATPase protein that is a target of SL signaling. When SL binds with D14, it promotes the interaction with D14, D3, and D53. This interaction eventually leads to the polyubiquitination of D53 and its subsequent degradation by the 26S proteasome pathway, which in turn is proposed to release the repression of downstream target genes (Jiang et al. 2013; Zhou et al. 2013).

TEOSINTE BRANCHED1 (*TB1*), which encodes a TCP family transcription factor, works downstream of SLs to inhibit the outgrowth of axillary buds in rice (Choi et al. 2012). *TILLERING AND DWARF 1* (*TAD1*) or *TILLER ENHANCER* (*TE*), which encodes a co-activator of the anaphase-promoting complex (APC/C), targets MOC1 for degradation in a cell-cycle-dependent manner to control rice tillering (Xu et al. 2012; Lin et al. 2012). Most recently, D14 has been defined as a noncanonical hormone receptor that mediates the hydrolysis of SL to a covalently linked intermediate molecule (CLIM) (Yao et al. 2016). Recently, IPA1 has been proposed and confirmed as one of the downstream targets of SLs in rice (Song et al. 2017).

8.4.4 Tiller Number and Rice Breeding

In rice, panicle number depends mainly on tiller number, and breeding cultivars with moderate- to high-tillering ability was an important objective. Tiller number is determined by bud number, and bud formation is determined by the SAM and AM. Mutants in SL biosynthesis and signaling are usually too extreme to be useful in plant breeding. However the *htd1* mutant encoding CCD7 (equivalent to *MAX3* in *Arabidopsis*) is an important QTL in rice (Qian et al. 2016). Furthermore, variants in rice with different complements of *MAX1* genes have different tiller numbers (Cardoso et al. 2014). Another important gene that influences tiller number is *IPA1* (Jiao et al. 2010) Fig. 8.2.

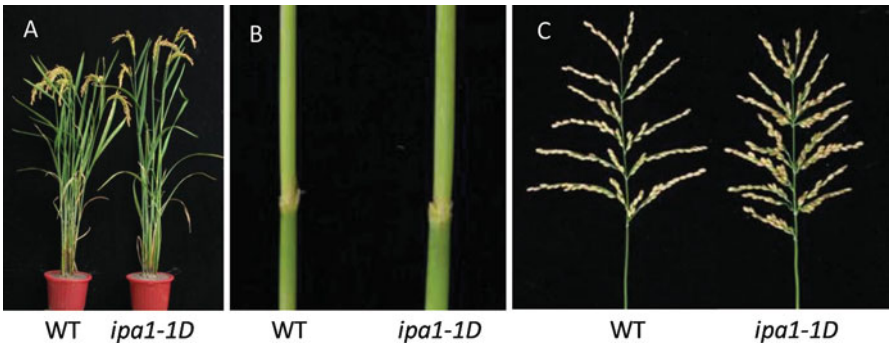

Fig. 8.2 Effects of the *ipa1* gene on tiller and panicle development. The *ipa1-1D* allele carries a dominant mutation which results in (**a**) taller stems and fewer tillers, (**b**) thicker stems, and (**c**) larger panicle with increased branches

Qian et al. (2007) introduced nine mostly unidentified tillering genes, but including IPA1, into the *indica* rice restorer line 93-11 to construct introgression lines (ILs) and chromosome segment substitution lines (CSSLs). The resultant CSSLs carry two to nine tillering genes through crossing various CSSLs carrying single tillering genes. The effects of the individual tillering genes were evaluated by analyzing their phenotypic contributions to generate a genotype-phenotype (G-P) database. Therefore, CSSLs can be selected to generate a specific number of tillers. For example, a tiller number ranging from 28 to 31 were developed in the line CSSL21, which contains five tillering genes that can be traced and manipulated by markers linked to each. Thus, by using proper combinations of tillering genes, a rice variety can be rationally designed and created to have the desired phenotype (Guo et al. 2008; Liu et al. 2012).

8.5 Tiller Angle

8.5.1 Importance of Tiller Angle

In rice the tiller angle, which is usually defined as the angle between the tiller and the main culm, plays a key role in determining rice plant architecture. The angle of a tiller from vertical will also determine the angle of its leaves. In rice breeding practice, moderate tiller angle can increase photosynthetic efficiency and improve lodging resistance. Although leaves arise at 180° to each other on the main stem, tillers do not maintain this strict geometry. The angle between different tillers, or their direction of growth, can also vary, and this will influence the shape or "bushiness" of the plant. However it is not known what factors or genes determine the direction in which tillers grow.

8.5.2 Genes Controlling Tiller Angle

The genetic study of the molecular mechanisms controlling rice tiller angle has lagged behind studies of tiller number. Only a few genes controlling tiller angle have been cloned, and the specific functions of these genes and the molecular regulatory mechanism are largely unknown. We know little about how these genes are coordinated or regulate each other.

The *PROSTRATE GROWTH 1* (*PROG1*) gene encodes a single Cys2-His2 zinc-finger protein, which was of central importance in the key transition from prostrate to erect growth in rice domestication (Tan et al. 2008; Jin et al. 2008). A major quantitative trait locus (QTL) controlling tiller angle in rice is *Tiller Angle Control 1* (*TAC1*). High expression of *TAC1* results in a wider tiller angle and low expression leads to erect tillers with a smaller tiller angle (Yu et al. 2007). A gene known as *LEAF and TILLER ANGLE INCREASED CONTROLLER* (*LIC*) encodes a CCCH-type zinc-finger protein, which is mainly involved in leaf angle regulation. Downregulation of *LIC* gene increases leaf and tiller angles (Wang et al. 2008). The *PLANT ARCHITECTURE AND YIELD 1* (*PAY1*) gene is a negative regulator of rice tiller angle. Overexpression of *PAY1* leads to small tiller angle, and downregulation of *PAY1* expression level results in greater tiller angle (Zhao et al. 2015). The *LOOSE PLANT ARCHITECTURE1* (*LPA1*) gene encodes a plant-specific INDETERMINATE DOMAIN (IDD) protein and acts as a transcriptional repressor which regulates tiller angle (Wu et al. 2013). Tiller angle in rice cultivars is largely controlled by *TAC1* and *TAC3* together with *D2* (Dong et al. 2016).

The *LAZY1* (*LA1*) gene, the first cloned gene that controls rice tiller angle, encodes a novel grass-specific protein that negatively regulates polar auxin transport (Li et al. 2007; Yoshihara and Iino 2007). Progress in understanding the molecular mechanism of tiller angle control has come from the in-depth study of *LA1*. The *lazy1 (la1)* mutant plants exhibit a tiller-spreading phenotype which appears to be caused by a significant increase in polar auxin transport and decrease in lateral auxin transport, resulting in a disturbance of the asymmetric distribution of auxin in the shoot base (Sang et al. 2014). A screen for *SUPPRESSORS OF la1* (*SOLS*) identified genes mutated in the SL signaling pathway. Such mutants showed that SLs inhibit auxin biosynthesis in the shoot base and negatively regulate shoot gravitropism (Sang et al. 2014).

8.6 Leaf Morphology

8.6.1 The Importance of Leaf Morphology

Rice leaf morphology is considered to be a key component of plant architecture and is characterized by leaf rolling, leaf angle, leaf drooping, and leaf width. Leaf

architecture plays an important role in determining grain yield in rice, because it determines canopy architecture, the effectiveness with which light is intercepted, and the provision of photosynthate to the panicle. The flag leaf is considered to be particularly important in grain filling since it provides much of the photosynthate.

8.6.2 Genes Controlling Leaf Rolling

There are two types of leaf rolling in rice, adaxial and abaxial rolling. There are mainly two mechanisms controlling leaf rolling. One is the turgor changes of bulliform cells, and the other is the development of specialized cells in the epidermis.

Bulliform cells are large, thin-walled, and highly vacuolated cells and play an important role in regulating leaf rolling. Several genes which are associated with bulliform cell development, such as *ABAXIALLY CURLED LEAF 1* (*ACL1*), *NARROW AND ROLLED LEAF 1* (*NRL1*), *Rice outermost cell-specific gene5* (*Roc5*), *SEMI-ROLLED LEAF1* (*SRL1*), *Rolling-leaf14* (*RL14*), *SHALLOT-LIKE2* (*SLL2*), *ROLLED and ERECT LEAF 2* (*REL2*), and the novel lateral organ boundaries domain (LBD) gene *OsLBD3-7*, have been characterized as leaf rolling genes in rice (Li et al. 2010b, 2016a; Hu et al. 2010; Zou et al. 2011; Xiang et al. 2012; Fang et al. 2012; Zhang et al. 2015; Yang et al. 2016). It has been shown that contraction or expansion of bulliform cells in the adaxial or abaxial side results in changes in the symmetry of leaves, which is often manifest as leaf curling.

A key gene controlling leaf rolling is *SHALLOT-LIKE1* (*SLL1*). This gene encodes a MYB family transcription factor belonging to the KANADI family. Deficiency in SLL1 leads to defective programmed cell death of mesophyll cells on the abaxial side (Zhang et al. 2009a). Rice *curly flag leaf1* (*cfl1*) mutant is a dominant curly leaf mutant. The *CFL1* gene encodes a WW (tryptophan—tryptophan) domain protein and is predominantly expressed in specialized epidermal cells. Reduced expression of *CFL1* resulted in reinforcement of cuticle structure and caused leaf curling (Wu et al. 2011).

8.6.3 Genes Regulating Leaf Angle

Research shows that BR promotes cell elongation at the adaxial side of the lamina joint, which increases leaf inclination (Zhang et al. 2014a). The key genes in BR biosynthesis (*BRD1*, *BRD2*, *D2*, *D4*, and *D11*) (Hong et al. 2002, 2003, 2005; Tanabe et al. 2005; Sakamoto et al. 2006) and signaling pathway (*D1*, *D61*, *BAK1*, *BZR1*, *DLT*, *GSK2*, and *TUD1*) (Yamamuro et al. 2000; Nakamura et al. 2006; Wang et al. 2006; Bai et al. 2007; Li et al. 2009a; Tong et al. 2012, 2009; Hu et al. 2013) also regulate leaf angle. New components of the BR signaling pathway also regulate leaf angle. The *INCREASED LEAF INCLINATION 1* (*ILI1*) gene encodes

an HLH protein which positively regulates leaf angle. In contrast, ILI1 Binding bHLH Protein 1 (IBH1) negatively regulates leaf angle. Both ILI1 and IBH1 are direct targets of BZR1, but they antagonize each other (Zhang et al. 2009b). The C3H-type transcription factor LIC1 antagonizes BZR1 to repress BR signaling in rice (Zhang et al. 2012a). Another gene, *BRASSINOSTEROID UPREGULATED 1* (*BU1*), is a BR-induced transcription factor which positively regulates leaf angle through the BR signaling pathway (Tanaka et al. 2009). Two further genes that regulate leaf angle are *LEAF INCLINATION 2* (*LC2*), which encodes a VIN3-like protein with conserved PHD and VID domains (Zhao et al. 2010), and *OsGSR1*, a member of the *GAST* family in rice, which is induced by GA and repressed by BR (Wang et al. 2009). Together, LC2 and OsGSR1 regulate leaf angle through the cross talk of BR with other hormonal signals (Wang et al. 2009; Zhao et al. 2010).

8.6.4 Other Genes Affecting Leaf Morphology

The *DROOPING LEAF* (*DL*) gene encodes a putative transcription factor containing a zinc-finger domain and YABBY domain and is required for the formation of the midrib in the leaf. A loss-of-function mutation in *DL* results in a leaf lacking a midrib resulting in drooping leaf phenotypes (Ohmori et al. 2011). A gene known as *NARROW AND ROLLED LEAF 1* (*NRL1*) or *NARROW LEAF AND DWARF 1* (*ND1*) encodes cellulose synthase-like protein D4 (OsCSLD4) (Li et al. 2009b; Hu et al. 2010). The OsCSLD4 protein affects leaf width through regulation of morphogenesis of the midrib vascular bundle. Leaf width is regulated through auxin biosynthesis and polar auxin transport mediated by *NARROW LEAF 1* (*NAL1*) and *NAL7* genes (Fujino et al. 2008; Qi et al. 2008).

8.7 Panicle Morphology

8.7.1 The Panicle

The inflorescence of rice is known as the panicle. It is a compound branched inflorescence with primary and secondary branches, terminating in spikelets. The panicle is a determinate structure produced by each stem. Therefore, growth of the rice plant stops upon transition from vegetative to flowering phases, and the timing of flowering is very important in the determination of plant architecture. The degree and pattern of panicle branching is important to determine the number of grains that will be produced. Thus panicle morphology is one of key agronomic traits closely related to grain yield and has always been an important part of rice ideal plant architecture. Panicle morphology is characterized as panicle length, spikelet number and density, and seed setting rate.

8.7.2 Important Panicle QTLs Cloned in Rice

Numerous genes have been identified that control panicle development. Genes that influence the timing of panicle development include *Ghd7* (*GRAIN NUMBER, PLANT HEIGHT, AND HEADING DATE 7*) encoding a CCT domain protein, which is considered to be a key regulator of heading date to enhance grain yield. Increased expression level of *Ghd7* delays heading date and prolongs the duration of panicle differentiation (Xue et al. 2008). The *Ghd7* gene also regulates tiller branching through phytochrome signaling (Weng et al. 2014). Another gene, *Ghd8*, encodes a putative Heme Activator Protein 3 (OsHAP3) subunit of a CCAAT-box binding protein and has a positive effect on both tiller number and primary and secondary branches by upregulating *MOC1* (Yan et al. 2011).

The *IDEAL PLANT ARCHITECTURE1* (*IPA1*) gene is one of the most important in the control of panicle size and also tiller number. It encodes a key transcription factor OsSPL14, which has pleiotropic effects in regulating plant architecture in rice. Gel shift assays show that IPA1 could directly bind to the promoter of *OsTB1* to suppress rice tillering (Lu et al. 2013). Research also shows that *IPA1* expression is regulated at the protein level by IPI1 (IPA1 Interacting Protein 1) or at the RNA level by OsmiR156 or DNA methylation of the IPA1 promoter sequence and *IPA1* regulates panicle length and plant height through *DEP1* (*DENSE AND ERECT PANICLE1*) (Jiao et al. 2010; Lu et al. 2013; Wang et al. 2017; Zhang et al. 2017). The *DEP1* gene is a major dominant QTL that leads to an erect panicle. The *DEP1* locus is a gain-of-function mutation resulting in an increase of primary and secondary branches and number of grains per panicle (Huang et al. 2009).

Elongation of rice panicle is influenced by *SHORT PANICLE 1* (*SP1*), which encodes a putative transporter that belongs to the peptide transporter (PTR) family (Li et al. 2009c). Elongation is also influenced by *DEP2* which encodes a plant-specific protein and is strongly expressed in young panicles, affecting the elongation of panicle branches (Li et al. 2010a).

Other key genes include *Grain number 1a* (*Gn1a*) which encodes cytokinin oxidase/dehydrogenase (OsCKX2), catalyzing the degradation of active cytokinin (CK). Reduced expression of *Gn1a* leads to accumulation of CK in inflorescence meristems (IM) and increases grain number per panicle, resulting in enhanced grain production (Ashikari et al. 2005). The *DROUGHT AND SALT TOLERANCE* (*DST*) gene encodes a zinc-finger transcription factor, which is shown to be a direct upstream activator of *Gn1a* expression in the reproductive meristem (Li et al. 2013). Thus, cytokinin plays an important role in regulating panicle development, but the molecular mechanism is unclear. Further control is mediated by miR172 and miR529 which regulate expression of their target genes (*AP2* and *SPL*, respectively) and hence regulate tiller and panicle branching (Wang et al. 2015b).

8.8 Grain Shape and Size

8.8.1 Rice Grains

While panicle branching determines the number of grains per tiller, the yield of grain is also determined by the size of the grain. The grain is a single-seeded fruit or caryopsis. Grain size or shape is also an important quality trait of rice grains because of the preferences of consumers in different geographical locations around the world. Different varieties have different shapes and sizes which may need to be maintained to satisfy consumer preferences. Grain size also affects cooking properties. Therefore simply increasing grain size might not be the optimum way to increase grain yield. Nevertheless it is a key yield trait. Grain size is often described by its three-dimensional structures: grain length (GL), grain width (GW), and grain thickness (GT) (Zuo and Li 2014).

8.8.2 Genes Regulating Grain Size

The *Grain size 3* (*GS3*) gene is a major QTL controlling both grain weight and grain length, with minor effects on grain width and thickness (Fan et al. 2006). This gene encodes a putative transmembrane protein containing a plant-specific organ size regulation (OSR) domain in the N terminus, which functions as a negative regulator of grain size (Mao et al. 2010). Another gene, *Grain size 5* (*GS5*) positively regulates grain width, filling, and weight by promoting cell division. The *GS5* gene encodes a putative serine carboxypeptidase and functions as a positive regulator of grain size (Li et al. 2011). The *Grain width 2* (*GW2*) gene encodes a RING-type protein with E3 ubiquitin ligase activity and negatively regulates cell division by recruiting its substrate(s) for proteasome degradation (Song et al. 2007). A major QTL controlling grain width and grain size is *Grain width 5* (*GW5*) which encodes a nuclear protein and physically interacts with polyubiquitin, suggesting that GW5 likely acts in the ubiquitin-proteasome pathway to regulate grain size (Weng et al. 2008). The *Grain weight 6a* (*GW6a*) gene encodes a new-type GNAT-like protein with intrinsic histone acetyltransferase activity and is also referred to as *OsgIHAT1*. High expression of *OsgIHAT1* enhances grain weight and yield through enlarging spikelet hulls (Song et al. 2015). A gene known as *Grain weight 7*, *Grain length 7*, or *Slender Grain 7* (*GW7*/*GL7*/*SLG7*) encodes a protein homologous to *Arabidopsis thaliana* LONGIFOLIA proteins. Upregulation of GW7/GL7/SLG7 expression leads to an increase in grain length via regulating longitudinal cell elongation (Wang et al. 2015a, c; Zhou et al. 2015).

Another gene, *Grain width 8* (*GW8*), encodes the SBP-family transcription factor OsSPL16, which is a positive regulator of cell proliferation. High expression level of *GW8* promotes cell division and grain filling, thus increasing grain width and yield (Wang et al. 2012). A plant-specific transcription factor OsGRF4 (Growth-Regulating Factor 4) is encoded by *Grain length 2*/*Grain size 2* (*GL2*/*GS2*) and is regulated by *OsmiR396*. Mutations in *GL2*/*GS2* affecting the binding site of *OsmiR396* increase expression level of *GL2*/*GS2*, resulting in increasing grain weight and yield (Hu et al. 2015; Duan et al. 2015; Che et al. 2015). *Grain length 3* (also named *GL3.1*) encodes a putative protein phosphatase with the Kelch-like repeat domain (OsPPKL1), which influences protein phosphorylation in the spikelet to increase cell division, thus increasing grain length and yield (Zhang et al. 2012b; Qi et al. 2012).

A very important QTL is *Thousand-grain weight 6* (*TGW6*) that encodes a novel protein with putative indole-3-acetic acid (IAA)-glucose hydrolase activity and positively regulates free IAA levels in grains, which reveals an important mechanism regulating grain filling during endosperm development (Ishimaru et al. 2013). Another gene, *SMALL GRAIN 1* (*SMG1*), encodes a mitogen-activated protein kinase kinase 4 (OsMKK4), which plays a role in cell proliferation, thus influencing grain size (Duan et al. 2014). *Big grain 1* (*BG1*) encodes a novel membrane-localized protein and overexpression of *BG1* leads to a significant increase in grain size by regulating auxin transport (Liu et al. 2015). *Big grain 2* (*BG2*) encodes a cytochrome P450 (OsCYP78A13) and variations in CYP78A13 coding region influence grain size and yield (Xu et al. 2015). Therefore, many genes and proteins have been identified that control grain architecture (size and shape), suggesting that many factors come into play to regulate grain development. This opens up the possibility to improve grain production through rational genetic design (see also Chap. 11).

8.9 The Application of Plant Architecture in Practical Rice Breeding

The progress made in functional genomics research is greatly accelerating rice molecular breeding by rational design. There are now several genes with a strong impact on plant architecture, including *sd1*, *IPA1*, and *GS3*, which have been applied to improve grain yield in rice. The rice *semi-dwarf 1* gene is well known as the “Green Revolution” gene, which has been used to reduce plant height to improve lodging resistance and considerably improve rice yield since the 1960s. The *sd1* gene together with associated improvements in crop production almost doubled the rice production in Asia between 1960 and 1998.

The use of the *IPA1* gene results in ideal plant architecture with reduced tiller number, increased panicle size, and thick and sturdy culms. It has been adopted as an effective way to increase grain yield. For example, introduction of the *ipa1* allele

into a *japonica* rice variety Xiushui11 (XS11) increased grain yield approximately 10% in test plots (Jiao et al. 2010), and the gene has subsequently been adopted more widely, with similar gains in yield (Qian et al. 2016; Zhang et al. 2017). Grain size is another important trait that affects rice yield. The gene *GS3* has a major effect on grain size, and natural variation exists widely at the *GS3* locus which has been widely used in rice breeding (Takano-Kai et al. 2009; Fan et al. 2009).

The increasing use of high-yielding F_1 hybrids provides an opportunity to readily combine genes that control architecture in novel or flexible combinations by choosing parental lines with particular genes. In this way F_1 varieties can be produced with architectural attributes suited to particular conditions or environments (Qian et al. 2016). A further development is the application of CRISPRR/Cas9 technology in plant genome editing. This will accelerate the transfer of results of functional genomic research into rice breeding practice. For example, the CRISPR/Cas9 system has been used to mutate the *IPA1*, *Gn1a*, *GS3*, and genes in rice cultivar Zhonghua 11, resulting in fewer tillers, larger panicles with increased numbers of flowers per panicle, and increased number and size of grains (Li et al. 2016b). Further advances are anticipated as new genes and alleles are identified and the interactions between genes are better understood. In the longer term, rational design will make increasing use of modeling approaches to better define the way in which yield is determined by architecture. Optimal architectures can then be identified and subsequently created by precision breeding.

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Chapter 9
Breeding Applications and Molecular Basis of Semi-dwarfism in Rice

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Abstract The green revolution in rice was defined by an unprecedented increase in rice production that saved the world from an impending famine in the 1960s. Driving this revolution was the *semi-dwarf 1* (*sd1*) gene conferring the semi-dwarf phenotype to the rice plant. The shorter stature conferred by *sd1* gives the plants resistance to lodging even under heavy doses of nitrogen fertilizer. IR8 carrying *sd1*, also known as the miracle rice, was the first high-yielding rice variety that came out as a result of intensive research and breeding efforts that capitalize on the use of the semi-dwarf trait to significantly improve rice yield. Although the rice green revolution gene has been used for breeding for decades, the *sd1* gene was not identified for a long time. Advancement of rice genomics facilitated the discovery that *SD1* encodes the GA biosynthesis gene, *GA20ox2*. Genome sequencing revealed that several of the varieties used as donor lines in breeding for the semi-dwarf phenotype in rice possess different alleles of *sd1*. Apart from breeding applications, dwarf mutants have also been instrumental in uncovering the molecular mechanisms underlying gibberellin biosynthesis and signaling.

Keywords Dwarfism · Green revolution · Gibberellin · *sd1* · Rice breeding

9.1 Introduction

Rice is one of the most important food crops, providing up to 23% of the daily caloric needs of the world population (GRiSP 2013). Archeological evidence dates the domestication of rice between 8000 and 10,000 years ago (Jiang and Liu 2006; Londo et al. 2006; Sweeney and McCouch 2007). During domestication, ancient farmers and gatherers selected naturally occurring variants of the wild progenitors

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of rice with desirable characteristics, including seed retention (i.e., non-shattering of grains), higher yield, and better grain quality. Generations of continuous selection for desirable traits slowly transformed wild rice into a cultivated variety or landrace with a distinct identity, genotype variability, and regional adaptation (Villa et al. 2006). Historically, the genetic changes that led to the establishment of cultivated rice resulted from selections that were geared toward making the wild rice species more suitable not only for human consumption but also for cultivation. Therefore, it is conceivable that ancient farmers selected shorter, non-lodging plants for easier gathering and harvesting. Indeed, evidence of artificial selection for the shorter phenotype during the early stages of domestication of *japonica* rice has been reported (Asano et al. 2011). In modern agricultural history, shorter plant stature had played a key role in increasing rice production, resulting in the establishment of many semi-dwarf rice varieties in many countries. After World War II, the global population began to increase exponentially, severely threatening food security. In an attempt to increase food production, particularly by increasing the grain yield of rice and wheat, chemical fertilizers were introduced into crop production systems (Smil 1999; Erisman et al. 2008). The application of chemical fertilizers generally increased grain yield, but it also induced the excessive growth of crops. These taller plants were more susceptible to being blown over by wind or lodged by rain, resulting in yield losses. Because of the excessive lodging associated with the use of chemical fertilizers (Ram 2014), different strategies of increasing grain yield had to be explored. Breeding for rice varieties that responds to high fertilization input without the excessive growth led to the development of semi-dwarf rice varieties.

In this chapter, we first introduce the history of semi-dwarf breeding in rice, highlighting the impact of the semi-dwarf phenotype in increasing yields during the green revolution in rice. Then, we describe *semi-dwarf 1* (*sd1*), the causal gene conferring the semi-dwarf phenotype in rice. The various *sd1* alleles that have been widely used in semi-dwarf breeding programs are also presented. The dwarf mutants of rice became instrumental not only in the identification of causal genes controlling the dwarf phenotype but also in the elucidation of the molecular basis and regulatory pathways involved in the expression of dwarfism (Table 9.1). Here, we also present the genetic regulators of gibberellin (GA) biosynthesis and signal transduction and how the specific interaction among these regulators can control plant height.

9.2 History of Semi-dwarf Breeding

Semi-dwarf breeding in rice using the *semi-dwarf 9* [*sd9*, *sd*(*t*)] allele started as early as the 1930s in Japan (Tanisaka et al. 1994). This allele has since been subsequently introgressed to several Japanese varieties (Tanisaka et al. 1994). Tan-Ginbozu, a shorter rice variety derived from natural variants of the variety Ginbozu, as well as the semi-dwarf variety Jikkoku, has also been widely used in

Table 9.1 List of dwarf mutants

Name	CGSNL gene name	Gene symbol synonym	Chr.	RAP ID	Protein name	Description
D1	Daikoku dwarf	d1, GPA1, GA1, RGA1, dwf1, XA7, RGA, D1/RGA1, D89	5	Os05g0333200	Guanine nucleotide-binding protein alpha-1 subunit	Guanine nucleotide-binding protein alpha-1 subunit (GP-alpha-1)
D2	Dwarf Ebisu	d2, dwf2, CYP90D2, D2/CYP90D2, OsD2, SMG11, OsSMG11, D2/SMG11	1	Os01g0197100	C-23 hydroxylase, cytochrome P450 90D2	Brassinosteroid biosynthesis. Cytochrome P450. Regulation of plant architecture
D3	Dwarf Bunketsuwaito tillering	d3, dwf3, D3/OsMAX2, Os_F0760, SOL1, OsORE9	6	Os06g0154200	F-box/LRR-repeat MAX2 homolog	F-box component of the SKP-Cullin-F box (SCF) E3 ubiquitin ligase complex. Strigolactone (SL) signal perception. Similar to F-box/LRR-repeat MAX2 homolog
D4	Dwarf Bunketsuwaito tillering	d4, dwf4	6			
DWARF4	Dwarf4	OsDWARF4, osdwarf4, OsDWF4, CYP90B2	3	Os03g0227700	Cytochrome P450 90B2	Brassinosteroid biosynthesis. Brassinosteroid C-22 hydroxylase
D5	Dwarf Bunketsuwaito tillering	d5, dwf5	2			
D6	Dwarf Ebisumochi dwarf or Tankanshirazasa	d6(d34), d34, dwf6	7			
D7	Dwarf Heieidaikoku or Cleistogamous	d7, dwf7	7			
D9	Dwarf Chinese	d9, dwf8	6			

(continued)

Table 9.1 (continued)

Name	CGSNL gene name	Gene symbol synonym	Chr.	RAP ID	Protein name	Description
D10	Dwarf Kikeibanshinriki or Toyohikaribunwai tillering	d10 (d15,d 16), d16, d15, dwf9, d10, OsCCD8b, OsCCD8, CCD8, OsD10	1	Os01g0746400	Carotenoid dioxygenase 8	Strigolactone biosynthetic process. Similar to carotenoid cleavage dioxygenase. One of MAX4 orthologues
D11	Dwarf Shinkaneaikoku or Nohrin 28	d11(d8), dwf10, d8, D11/CYP724B1, d11, CYP724B1	4	Os04g0469800	Cytochrome P450 724B1	Brassinosteroid biosynthesis, cytochrome P450
D12	Dwarf Yukara dwarf or Fukei 71	d12 (d50), dwf11, d50, d12, d32, F71, DMT9	2	Os02g0477700	Inositol polyphosphate 5-phosphatase	D50 gene, encoding inositol polyphosphate 5-phosphatase, is required for development of intercalary meristem (Sato-Izawa et al. 2012)
D13	Dwarf short grained	d13, dwf12				
D14	Dwarf Kamikawabunwai tillering	d14 (d10), dwf13, d10, d14, D88, HTD2, OsD14, qPPB3, D88/D14	3	Os03g0203200	Alpha/beta-hydrolase	Strigolactone receptor
D17	Dwarf slender dwarf, tillering	d17(t), dwf14, d17, htd1, OsCCD7, CCD7, D17/HTD1, HTD1, OsD17	4	Os04g0550600	Carotenoid dioxygenase 7	Strigolactone biosynthetic process. MAX3 ortholog, carotenoid cleavage dioxygenase 7
d18h	Hosetsu-waisei	d18h (d18-I, d18h), dwf15, d25, d18, d18-k, d18-AD, d18-dy, OsGA3ox2, ga3ox2, OsGA3OX2, GA3OX2, GA3ox-2, GA3ox1	1	Os01g0177400	Gibberellin(GA)-3 beta-hydroxylase 2	Relating to gibberellin biosynthesis. Gibberellin(GA)-3 beta-hydroxylase-2 (GA3ox-2) catalyzes the reaction from GA20 to GA1 and GA9 to GA4
d18-AD	Akibare-waisei					
d18-dy	Waito-C					
d18k	Kotaketamanishiki					
D19	Dwarf Kamikawa	d19(t), dwf16, d19				
D20	Dwarf Hayayuki	d20, dwf17	3			
D21	Dwarf Aomorimochi-14	d21, dwf18	6			

D22	Dwarf Jokei 6549	d22(t), dwf19, d22				
D23	Dwarf Ah-7	d23(t), dwf20, d23				
D24	Dwarf M-7	d24(t), dwf21, d24				
D26	Dwarf 7237	d26, dwf22	1			
D27	Dwarf Bunketsuto tillering	d27(d t), dt, dwf23, d27, OsD27	11	Os11g0587000	Beta-carotene isomerase	Strigolactones biosynthesis. Iron-containing protein, beta-carotene isomerase. OsDWARF27 may contribute to the formation of a-carotene-based strigolactone-like compounds (Bruno and al-Babili 2016)
D28	Dwarf Chokeidaikoku or long stemmed	d28(d C), dwf24, dC, d28	11			
D29	Dwarf short uppermost internode	d29(d K1), dwf25, dK1, d29	2			
D30	Dwarf Waiseishirasasa	d30(d W), dW, dwf26, d30	2			
D31	Dwarf Taichung 155 irradiated	d31, dwf27	4			
D32	Dwarf Kyushu 4	d32(d K4,d12), d12, dwf28, dK4, d32	2			
D33	Dwarf Bonsaito	d33(d B), dB, dwf29, d33	12			
D35	Dwarf Tanginbozu	d35, d35(t), dwf30, OsKOL2, KOL2, KO2, OsKO2, OSKO2, KO, CYP701A, CYP701A6, Os KO2, OsKOL2/CYP701A	6	Os06g0570100	Ent-kaurene oxidase-like protein 2	Relating to gibberellin biosynthesis. *ent*-kaurene oxidase (KO) catalyzes the reaction from *ent*-kaurene to *ent*-kaurenoic acid
D42	Dwarf Liguleless	d42, dwf31	4			
D49	Dwarf Reimei	d49(t)				

(continued)

Table 9.1 (continued)

Name	CGSNL gene name	Gene symbol synonym	Chr.	RAP ID	Protein name	Description
D51	Dwarf Kyushu 8	d51(dK8), dwf32, dK8, d51	8			
D52	Dwarf Kyushu 2	d52 (K2), dK2, dwf33, d52	3			
D53	Dwarf Kyushu 3	D53(D K3), DK3, Dwf34	11	Os11g0104300	Substrate of SCF-D3 ubiquitination complex	Dominant dwarf gene with many tillers. A repressor of strigolactone signaling. a substrate of the SCFD3 ubiquitination complex
D54	Dwarf Kyushu 5	d54(d K5), dK5, d54, dwf35	1			Dwarf induced by chemical mutagen on "Kinmaze" variety
D55	Dwarf Kyushu 6	d55(d K6), dK6, dwf36, d55	1			Dwarf induced by chemical mutagen on "Kinmaze" variety
D56	Dwarf Kyushu 7	d56(d K7), dwf37, dK7, d56	3			Leaf shape is similar to "daikoku dwarf," but plant type is not elect as it is. Grain shape is almost round but not so small as "daikoku dwarf"
D57	Dwarf	d57[d(x)], dwf38, d(x), d57	9			
D58(t)	Dwarf small grained	d58, dwf39	6			
D59(t)	Dwarf DM107-4	d59(t), dwf40, d59				
D60	Dwarf Hokuriku 100	d60 (sd(t)), sd(t), dwf41, d60	7			
sd1-d (sd1–1)	Semi-dwarf 1 (Dee-geo-wee-gen)	sd1, sd1 (d47, sd1-d, sd1-1), d49 (sd1-r), d47, C20OX2, OsGA20ox-2, OsGA20ox2, GA20ox-2, GA20OX2, GA20ox2, sd-1, SD1-ja, SD1-in, sd1-2, Sd-1, GA20, 20ox2, Os20ox2	1	Os01g0883800	Gibberellin 20-oxidase 2	sd1-d is a dwarf gene of Taiwanese cultivar "Dee-Geo-Woo-Gen"
sd1–2	Semi-dwarf 1 (Jikkoku)					Japanese native variety
sd1-c (sd1–3)	Semi-dwarf 1 (Calrose)					Dwarfing gene obtained from Calrose treated by gamma-ray. Effect of dwarfness is equal to or less than sd1-d

sd1-r (sd1–4)	Semi-dwarf 1 (Reimei)					Dwarfing gene obtained from Calrose treated by gamma-ray
sd-2	Semi-dwarf 2	sd2				
sd-3	Semi-dwarf 3	sd3				
sd-4	Semi-dwarf 4	sd4				
sd-5	Semi-dwarf 5	sd5				
Sd-6	Semi-dwarf 6	sd6				
Sd-7	Semi-dwarf 7	sd7, sd7(t)				
Sd-8	Semi-dwarf 8	sd8(t), sd8				
Sd-9	Semi-dwarf 9, Ginbozu	sd9(t), sd9, sd(t)				Origin of semi-dwarfism of Koshihikari
Sd-10	Semi-dwarf 10, Kinmaze	sd10(t), sd10				
SDG	Semi-dwarf (BRGPC)	sdg, sd11				

Modified from Matsuo et al. (1997)

semi-dwarf breeding. Jikkoku was later used to develop the semi-dwarf rice variety Shiranui.

In Taiwan, Taichung (Native) 1 was bred as a semi-dwarf rice variety from the short Taiwanese landrace Dee-Geo-Woo-Gen. Taichung (Native) 1 was single-handedly responsible for increasing the grain yield of rice in Taiwan (Huang et al. 1972) and was used as a donor line for numerous semi-dwarf and high-yielding rice varieties in tropical Asia and Korea. By 1970, the cultivation of several Tong-il-type, semi-dwarf varieties derived from crossing *japonica-indica* varieties with either Taichung (Native) 1 or IR8 as semi-dwarf genetic resources was already widespread in Korea (Shin and Shim 1975; OECD 2016).

Similar efforts to incorporate the semi-dwarf phenotype in traditional rice varieties were recorded in China in the 1950s. Ai-Jiao-Nan-Te was selected as a semi-dwarf variety from natural variants of Nan-Te 16 in 1956. Guang-Chang-Ai, on the other hand, was a semi-dwarf progeny derived from crosses between Ai-Zai-Zhan 4 and Guang-Chang 13. Zhen-Zhu-Ai was also selected as semi-dwarf progeny of Ai-Zai-Zhan 4. Ai-Jiao-Nan-Te, Guang-Chang-Ai, and Zhen-Zhu-Ai have since been successfully used as donor parents for numerous semi-dwarf varieties in China (IRRI and CAAS 1980; Qian and Liu 1993).

In the early 1960s, crosses made between the tall and good-tasting Indonesian rice variety Peta and the semi-dwarf Taiwanese landrace Dee-Geo-Woo-Gen at the International Rice Research Institute (IRRI) led to the development of the miracle rice, IR8 (Hargrove and Cabanilla 1979; Dalrymple 1986). IR8 has a semi-dwarf phenotype that allows the plant to channel its energy toward producing more grains instead of elongating upon application of nitrogen fertilizer. It also has erect leaves that give the plant better light-intercepting characteristics (Duncan 1971). IR8 doubled the yield of rice and served as the catalyst of the green revolution that averted impending famines in many countries in Asia.

Although IR8 offered high yields in combination with good cultivation practices, it had poor resistance to a suite of plant diseases and pests and low adaptability to low-fertility soils (IRRI 1976; Datta et al. 1968). To stabilize rice production in tropical and subtropical Asia, IRRI scientists screened a vast number of rice genetic resources available in their gene bank to identify sources of genes for biotic and abiotic stress resistance and incorporated them into IR8 via conventional breeding. The result was IR36, a new semi-dwarf, early flowering rice variety with multiple resistance to diseases (i.e., blast, bacterial blight, grassy stunt virus, and tungro virus) and pests (i.e., stem borer, gall midge, and brown and green leafhopper) and high adaptability to low-fertility soil (IRRI 1985; Khush et al. 2001; Guimaraes 2009). IR36 was released in 1976 and was distributed across Asia, including the Philippines, Vietnam, Indonesia, India, Malaysia, Cambodia, Laos, Bangladesh, and Sri Lanka. From 1966 to 1999, the adoption of these varieties doubled rice production in Asia (Khush 1999, 2001). Until now, IRRI is still producing semi-dwarf rice varieties carrying the *sd1* gene, underlining the importance of *sd1* in rice breeding.

9.3 Variants of the *sd1* Allele Used for Semi-dwarf Breeding in Rice

The recessive, semi-dwarf gene, *sd1*, from the Taiwanese rice landrace Dee-Geo-Woo-Gen has been identified as the causal gene conferring the semi-dwarf phenotype in Taichung (Native) 1, IR8, and their derivative varieties. Physiological and chemical analyses suggested that the semi-dwarf phenotype of *sd1* is related to a deficiency in the biosynthesis of active gibberellic acid (GA) (He and Li 1996). This was confirmed by subsequent genetic and biochemical analyses showing that *SD1* encodes the rice Gibberellin 20 oxidase-2 (GA20ox-2) (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002; Asano et al. 2007). An investigation of semi-dwarf varieties from China, the United States, and Japan showed that almost all of these varieties possess different *sd1* alleles, indicating that the *sd1* mutation has been widely used for semi-dwarf breeding (Fig. 9.1) (Futsuhara et al. 1967; Foster and Rutger 1978; Asano et al. 2007). The *sd1* allele of IR8 contains a 383-bp deletion from exon 1 to exon 2, causing a frameshift mutation that leads to a premature stop codon. This makes the *sd1* allele of IR8 a null allele (Fig. 9.1) (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002; Asano et al. 2007). Meanwhile, the Japanese variety Jikkoku has a single-nucleotide polymorphism (SNP) and an amino acid substitution (G94 V) in exon 1 (Fig. 9.1). The *sd1* allele of the Chinese variety Ai-Jiao-Nan-Te has a 2-bp deletion in exon 1, leading to a frameshift mutation that induces a premature stop codon (Fig. 9.1). The γ-ray-induced varieties Reimei and Calrose76 both carry a SNP in exon 3 and exon 2, leading to the amino acid substitutions D349H and L266F, respectively (Fig. 9.1). Zhayeqing8 has a SNP and amino acid substitution (P240L) in exon 2 (Fig. 9.1). The *sd1* allele of the Chinese variety 93-11 has a SNP that leads to a premature stop codon in exon 3 (Fig. 9.1). In addition, the *japonica* variety Nipponbare has a functional nucleotide polymorphism in *SD1* (100E and 340Q), resulting in lower enzymatic activity than the *SD1* in the *indica* variety Kasalath (100G and 340R) (Fig. 9.1) (Asano et al. 2011). Although numerous dwarf mutants have been reported (Table 9.1), it is interesting to note that different alleles of the same *SD1* gene have been used in independent semi-dwarf breeding programs across the globe. One possible reason for this is the unfavorable phenotype of other dwarf mutants (i.e., severe dwarfism, short panicle length, and small or sterile grains resulting in decreased yield) that makes them unsuitable for breeding. In contrast, all *sd1* mutants confer only the semi-dwarf phenotype without adverse pleiotropic effects on yield.

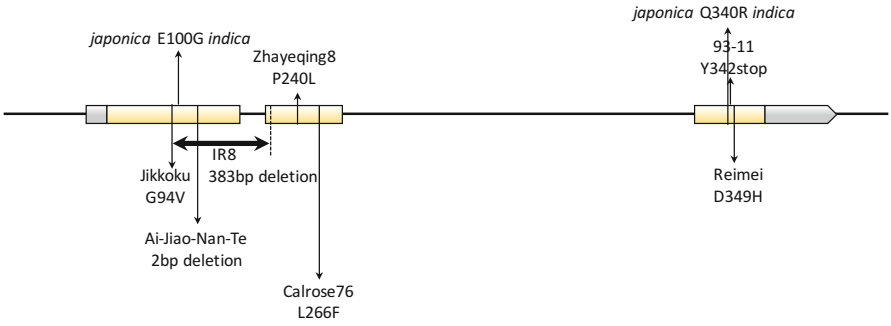

Fig. 9.1 Structure of the *SD1* gene and mutation site in semi-dwarf varieties. Gene structure of *SD1* (*GA20ox2*). The *GA20ox2* gene encoding GA 20-oxidase consists of three exons. Single-nucleotide polymorphism or deletion sites are indicated by *arrows*

9.4 Contribution of Dwarf Mutants to Biological Studies in Rice

9.4.1 GA Biosynthesis and Semi-dwarf Breeding

Bioactive GAs control many aspects of plant growth and development, including seed germination, stem and leaf elongation, and flower and seed development. Although over 100 GAs have been identified, only a small number of GAs, such as GA_1 and GA_4, are bioactive in plants (Yamaguchi 2008). The first step in GA biosynthesis involves the conversion of geranylgeranyl diphosphate into the tetracyclic hydrocarbon intermediate *ent*-copalyl diphosphate and then into *ent*-kaurene by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), respectively (Fig. 9.2). Both CPS and KS are members of the terpene synthase family. Subsequently, *ent*-kaurene is converted into GA_{12} by the two cytochrome P450 monooxygenases (P450), *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO), in the plastid membrane and endoplasmic reticulum, respectively (Fig. 9.2). Then, GA_{12} is converted into the bioactive form GA_4 by GA20ox and GA 3-oxidase (GA3ox), both of which are 2-oxoglutarate-dependent dioxygenases. GA_{12} is also converted into the precursor GA_{53} by GA 13-oxidase (GA13ox) (Table 9.2) (Magome et al. 2013), which is then converted into the bioactive form GA_1 by GA20ox and GA3ox (Fig. 9.2).

The rice genome possesses at least four *GA20ox* genes (*GA20ox1–GA20ox4*) (Table 9.2). *OsGA20ox2* is dominantly expressed in the stems, along with *OsGA20ox1* and *OsGA20ox4*. Therefore, mutation in *osga20ox2* as in the case of *sd1* can be partially compensated by *OsGA20ox1* and *OsGA20ox4*, resulting in the production of moderate amounts of GA in the stem (Itoh et al. 2004; Sakamoto et al. 2004).

All loss-of-function mutants for GA biosynthesis have been identified in rice, and all of the null mutants except for *GA13ox* and *GA20ox* genes showed a severe

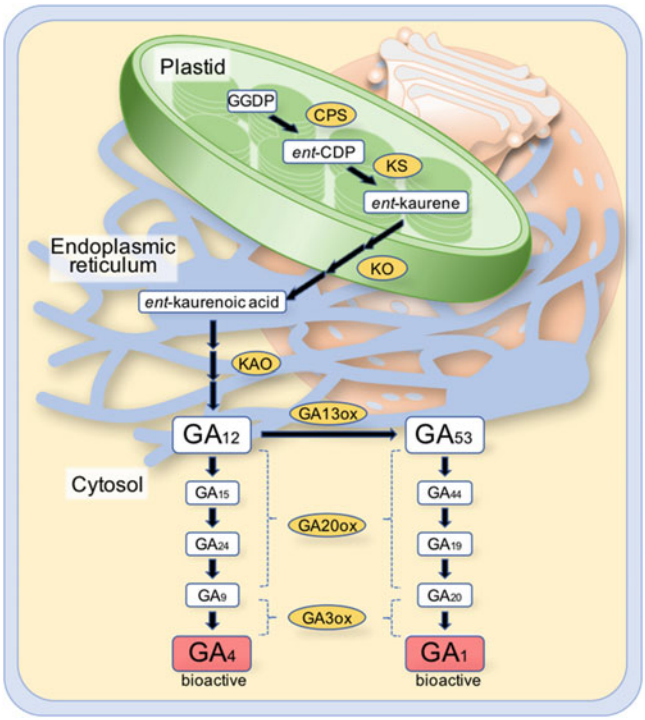

Fig. 9.2 Gibberellin biosynthesis pathway and predicted subcellular localization of metabolic enzymes. In plastids, geranylgeranyl diphosphate (*GGDP*) is converted into *ent*-kaurene by *ent*-copalyl diphosphate synthase (*CPS*) and *ent*-kaurene synthase (*KS*). *ent*-kaurene is converted to GA_{12} by *ent*-kaurene oxidase (*KO*) and *ent*-kaurenoic acid oxidase (*KAO*) in the endoplasmic reticulum. GA_{12} is converted into GA_{53} by GA13ox, and both GA_{12} and GA_{53} are converted into GA_9 and GA_{20}, respectively, by GA20ox. GA3ox catalyzes the biosynthesis pathway from GA_9 and GA_{20} into bioactive GA_1 and GA_4, respectively

dwarf phenotype (Sakamoto et al. 2004; Magome et al. 2013). Although the weak allele of KO (*d35*$^{\text{Tan-Ginbozu}}$) and GA3ox2 (*d18*-dy and *d18*k) confer a semi-dwarf phenotype, these semi-dwarf varieties have not been used widely. Why were mutations in *GA20ox2*/*SD1* selected over many other semi-dwarf mutants for breeding? Previous studies examined the suitability of *sd1* alleles in semi-dwarf breeding based on its internode elongation pattern relative to that of other semi-dwarf mutants (Itoh et al. 2004). Generally, four or five internodes elongate after the rice plant shifts from the vegetative to the reproductive stage. *sd1*, *d18*k, and *d35*$^{\text{Tan-Ginbozu}}$ mutants have shorter internodes than the normal rice plant although the internode elongation pattern differs between these mutants (Sakamoto et al. 2004). In the *sd1* and *d18*k mutants, the lower internode is shorter compared with the upper internode. On the contrary, the upper internode of *d35*$^{\text{Tan-Ginbozu}}$ mutant is shorter compared with the lower internodes. Plants with shorter lower internodes (*sd1* and *d18*k) would have a lower center of gravity compared to plants of the same height

Table 9.2 List of genes related to gibberellin biosynthesis in rice

Name	Gene symbol	Gene name		Gene locus	Descriptions
CPS	*ent*-copalyl diphosphate synthase	*OsCPS1*		Os02g0278700	GA biosynthesis
		OsCPS2		Os02g0571100	Involved in the biosynthesis of diterpene phytoalexins
		OsCPS3		Os09g0319800	Pseudogene
		OsCPS4		Os04g0178300	Involved in the biosynthesis of diterpene phytoalexins
KS	*ent*-kaurene synthase	*OsKS1*		Os04g0611800	GA biosynthesis
		OsKS2		Os04g0612000	
		OsKS3		Os04g0611700	
		OsKS4		Os04g0179700	Involved in the biosynthesis of diterpene phytoalexins
		OsKS5		Os02g0571300	
		OsKS6		Os02g0571800	
		OsKS7		Os02g0570400	Involved in the biosynthesis of diterpene phytoalexins
		OsKS8		Os11g0474800	Involved in the biosynthesis of diterpene phytoalexins
		OsKS9			Pseudogene
KO	ent-kaurene oxidase	*OsKO1*		Os06g0569900	Group 1, cytochrome P450 701A7, GA biosynthesis (Itoh et al. 2004)
		OsKO2	/ *D35*	Os06g0570100	Group 1, cytochrome P450 701A6, GA biosynthesis
		OsKO3			Group 1, pseudogene. Truncated form of N terminus (Itoh et al. 2004)
		OsKO4		Os06g0569500	Group 2, cytochrome P450 701A8, *OsKO4* does not rescue the severe dwarfism of osko2 mutant
		OsKO5		Os06g0568600	Group 2, cytochrome P450 701A9
KAO	*ent*-kaurenoic acid oxidase	*OsKAO*		Os06g0110000	GA biosynthesis
GA13ox	GA 13-oxidase	*OsGA13ox*		Os07g0681300	GA biosynthesis, cytochrome P450 714B1 (Magome et al. 2013)
		OsGA13ox		Os03g0332100	GA biosynthesis, cytochrome P450 714B2 (Magome et al. 2013)
				Os12g0118900	Not involved in GA biosynthesis, cytochrome P450 714C1 (Magome et al. 2013)
				Os12g0119000	Not involved in GA biosynthesis, cytochrome P450 714C2 (Magome et al. 2013)
				Os11g0119200	Not involved in GA biosynthesis, cytochrome P450 714C3 (Magome et al. 2013)

(continued)

Table 9.2 (continued)

Name	Gene symbol	Gene name		Gene locus	Descriptions
GA20ox	GA 20-oxidase	*OsGA20ox1*		Os03g0856700	GA biosynthesis, 2-oxoglutarate-dependent dioxygenase
		OsGA20ox2	/ *SD1*	Os01g0883800	GA biosynthesis, 2-oxoglutarate-dependent dioxygenase
		OsGA20ox3		Os07g0169700	GA biosynthesis, 2-oxoglutarate-dependent dioxygenase
		OsGA20ox4		Os05g0421900	GA biosynthesis, 2-oxoglutarate-dependent dioxygenase
GA3ox	GA 3-oxidase	*OsGA3ox1*		Os05g0178100	GA biosynthesis, 2-oxoglutarate-dependent dioxygenase
		OsGA3ox2	/ *D18*	Os01g0177400	GA biosynthesis, 2-oxoglutarate-dependent dioxygenase

Modified from Sakamoto et al. (2004)

(*d35*$^{\text{Tan-Ginbozu}}$), giving the former more resistance to lodging. Although *sd1* and *d18*k mutants exhibited similar internode elongation patterns, each internode of the *d18*k mutant was shorter than that of the *sd1* mutant (Itoh et al. 2004), making it unsuitable for breeding. This explains the prevalent use of the defective or mutant *ga20ox2*/*sd1* such as those found in Dee-Geo-Woo-Gen and its progenies, including IR8, in rice breeding.

9.4.2 GA Signaling Studies Using Rice Dwarf Mutants

Rice dwarf mutants not only played a key role in improving rice yields but also contributed in advancing our understanding of the molecular mechanisms underlying phytohormone signaling. The main pathway in GA signaling has been elucidated mostly based on rice dwarf mutant studies. This is in contrast to other phytohormone signaling models which had been constructed mainly using *Arabidopsis* mutants. This section outlines how plants perceive GA and transmit signals to induce the GA response.

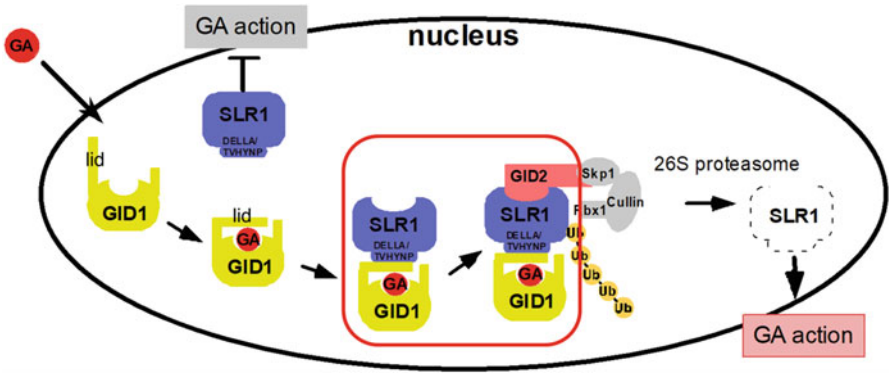

Fig. 9.3 Current model of gibberellin signaling (see text for details). Figure adapted and modified from Hirano et al. 2008

GA-deficient and GA-insensitive rice mutants are dwarf because GA promotes plant height. Determination of the causal genes for short stature from GA-insensitive rice mutants led to the identification of GA signaling components, GA INSENSITIVE DWARF1 (GID1) and GA INSENSITIVE DWARF2 (GID2) (Ueguchi-Tanaka et al. 2005; Sasaki et al. 2003). Another GA signaling component, SLENDER RICE1 (SLR1), was identified as the causal gene conferring the elongated and slender phenotype in *slr1* mutants (Ikeda et al. 2001). SLR1 belongs to the DELLA protein family and is the key repressor of rice GA signaling.

Extensive studies on the interaction of GID1, GID2, and SLR1 have established the early events of GA signaling in rice. In the absence of GA, GID1 and SLR1 do not interact, and SLR1 represses GA signaling (Fig. 9.3) (Hirano et al. 2008). When bioactive GA is synthesized in rice, the GA receptor GID1 perceives GA, which enables GID1 to interact with SLR1. Subsequently, GID2, an F-box protein that forms a complex with Skp and Cullin, specifically recognizes SLR1 within the GA-GID1-SLR1 complex and adds a polyubiquitin chain to SLR1. The SLR1 is then targeted for degradation via the 26S proteasome pathway. Elimination of SLR1 triggers various GA responses such as promotion of plant height (Hirano et al. 2008). All flowering plants studied up to now possess a similar GA-GID1-DELLA system. As mentioned above, GID1 and GID2 were initially identified from rice dwarf mutants, making them the starting point in elucidating the molecular mechanisms underlying GA action. The succeeding section will briefly describe the functions of GID1 and SLR1.

9.5 GID1 Protein

Rice contains a single GID1 (Ueguchi-Tanaka et al. 2005). Consistent with the current GA model, the rice *gid1* mutant exhibits severe dwarfism and a high degree of SLR1 accumulation (Ueguchi-Tanaka et al. 2005). Binding of GID1 with GA has

been demonstrated in crystal structure study (Shimada et al. 2008). When GA binds to the GA-binding pocket of GID1, the structurally flexible N-terminal strand of the GID1 protein associates with GA, which functions somewhat like a lid to cover the GA-binding pocket (Fig. 9.3). This coverage is thought to enable the outer surface of the GID1 lid to bind to the N-terminal DELLA/TVHYNP motif of SLR1, explaining why the GID1-SLR1 interaction occurs only in the presence of GA.

9.6 DELLA Proteins

Rice has a single DELLA protein, SLR1, whereas *Arabidopsis* has five (RGA, GAI, and RGL-1 to -3), and all of them function to repress GA signaling (Itoh et al. 2003). DELLA proteins belong to a plant-specific GRAS family of transcription regulators possessing a GRAS domain (Bolle 2004). In addition to the GRAS domain, DELLA proteins have a unique N terminus with two conserved motifs, the DELLA motif and the TVHYNP motif. Both DELLA and TVHYNP motifs are prerequisites for the GID1-DELLA interaction, and mutation in either motifs diminishes the interaction of DELLA with GID1. Mutation in the DELLA or the TVHYNP motif in DELLA proteins is not targeted for degradation, and this leads to severe dwarf phenotype (Ueguchi-Tanaka et al. 2007). On the other hand, mutations in the conserved amino acids of the GRAS domain often result in thin and elongated plant phenotype, demonstrating that the GRAS domain of DELLA proteins functions to repress GA signaling (Hirano et al. 2012).

9.7 Targets of DELLA Protein

Until recently, the molecular mechanisms governing the repression of GA signaling by DELLA proteins were unknown. However, it is now revealed that DELLA proteins interact with various transcription factors (TFs), and through these interactions, DELLAs regulate the transcription of genes involved in GA response (Daviere and Achard 2016). Several DELLA targets have already been identified in *Arabidopsis* and only a few in rice. Since the DELLA regulatory mechanism in rice and *Arabidopsis* are assumed to be similar in many cases, some examples of *Arabidopsis* DELLA targets will be presented below.

Hypocotyl elongation in *Arabidopsis* is promoted by GA and PHYTOCHROME-INTERACTING FACTORS (PIFs) (Alabadí et al. 2004). In 2008, two research groups identified how GA and PIFs regulate hypocotyl growth. DELLAs-PIF3/PIF4 interactions block PIF3/PIF4 from binding to their target gene promoter (Fig. 9.4a left) (de Lucas et al. 2008; Feng et al. 2008). This effectively inhibits the expression of genes targeted by PIF3/PIF4, resulting in the repression of hypocotyl growth. In the presence of GA, DELLAs are degraded and PIF3/PIF4 is released from such interaction. PIF3/PIF4 then regains its transcriptional activity to

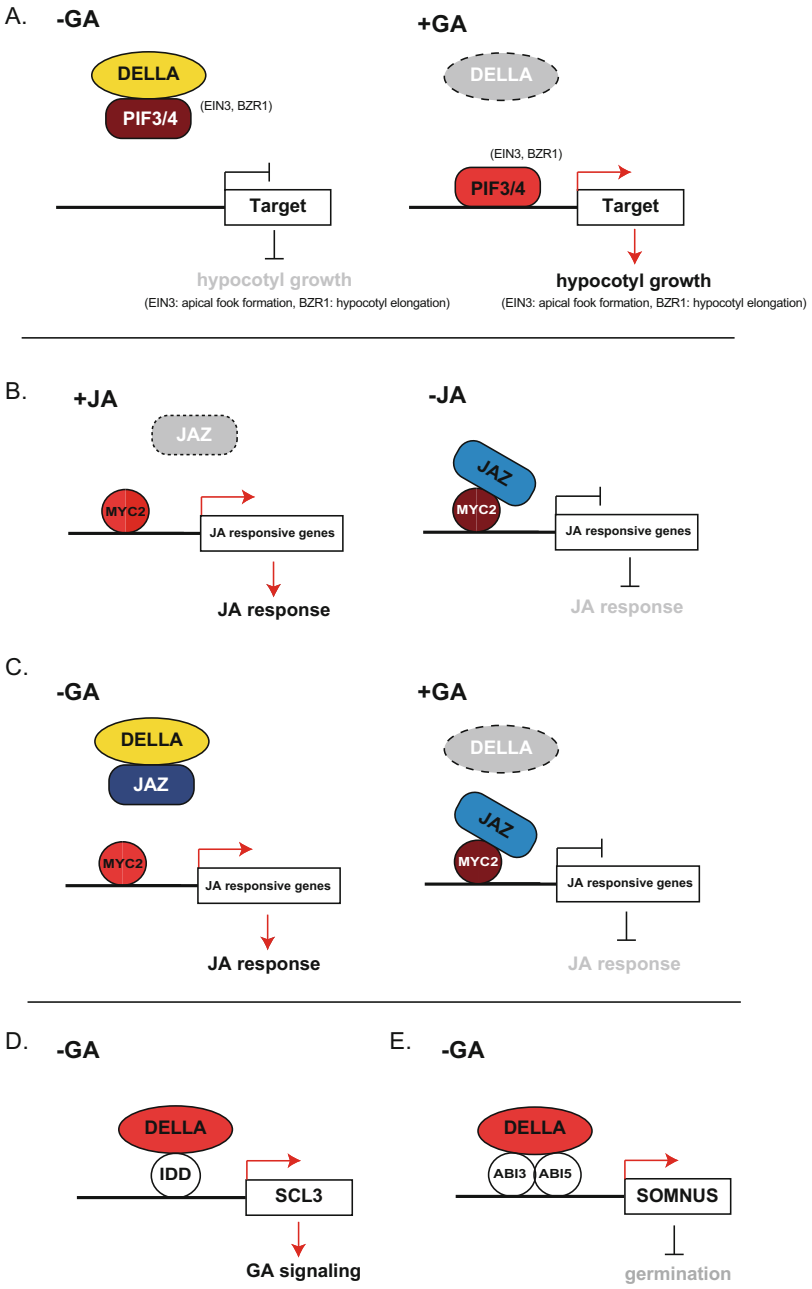

Fig. 9.4 Two mechanisms of DELLA protein function. (**a**–**c**) DELLA transcription factor (*TF*) trapping mechanism. In the absence of gibberellin (*GA*), DELLA protein interacts with and inhibits the function of the interacting TFs. In the presence of GA, the degradation of DELLA releases TFs, and TFs are activated. (**d**, **e**) DELLA interacts with TFs and promotes the transcription of target genes. In the presence of GA, DELLA is degraded, and transcription of target genes is repressed

promote hypocotyl elongation (Fig. 9.4a right). Another example of TF trapping by DELLAs is shown in the interaction between DELLAs and the repressor of jasmonate (JA) signaling, JA ZIM-domain proteins (JAZs). The basic helix-loop-helix transcription factor, MYC2, promotes the transcription of JA-responsive genes that promote JA signaling in *Arabidopsis* (Fig. 9.4b left). In the absence of JA, JAZs interact with MYC2, effectively inhibiting its transcriptional function and suppressing JA signaling (Fig. 9.4b right). In the presence of JA, JAZs are degraded via the 26S proteasome pathway, consequently MYC2 is released, and JA signaling promotes (Fig. 9.4b left, Hou et al. 2010). Under various JA levels, the interaction between DELLAs and JAZs releases MYC2 from its association with JAZs, allowing MYC2 to activate the expression of JA-responsive genes (Fig. 9.4c left). When DELLAs are degraded by GA, MYC2 interacts with JAZs, and JA signaling is suppressed (Fig. 9.4c right). These molecular mechanisms explain the cross talk between GA and JA signaling (Hou et al., 2010).

TF trapping by DELLAs has also been reported for other cases (Fig. 9.4a). For example, DELLAs can repress ethylene-induced apical hook formation by interacting with and inhibiting the function of ETHYLENE INSENSITIVE3 (EIN3), an ethylene signaling activator (An et al. 2012). In addition, brassinosteroid (BR)-induced hypocotyl elongation is inhibited via the interaction of DELLA with BRASSINAZOLE-RESISTANT1 (BZR1), a BR signaling activator (Bai et al. 2012). In summary, DELLAs repress GA response by interacting with TFs involved in light, JA, ethylene, and BR signal pathways.

Apart from TF trapping, DELLAs also play a role in transcriptional activation. DELLAs possess strong transcriptional activity; however, DELLAs are assumed not to directly bind to DNA (Bolle 2004; Hirano et al. 2012). In the simplest model, DELLAs should interact with DNA-binding TFs to activate DELLA target genes. Yoshida et al. (2014)) identified such DELLA-interacting TFs belonging to an INTERMEDIATE DOMAIN (IDD) protein family and showed that DELLA-IDD interactions can fine-tune GA homeostasis in *Arabidopsis*. Specifically, AtIDD3, AtIDD4, AtIDD5, AtIDD9, and AtIDD10 interact with the *Arabidopsis* DELLA protein RGA to enhance the transcription of SCARECROW-LIKE3 (SCL3) (Fig. 9.4d). SCL3 protein promotes root and hypocotyl elongation and radial patterning (Zhang et al. 2011). Although it may appear to be counterintuitive that RGA-IDD interactions induce the GA response via SCL3, this prevents the excess suppression of GA signaling by RGA, which is assumed to be a mechanism that maintains GA homeostasis. Similarly, DELLAs interact with ABI3/ABI5 in the promoter region of *SOMNUS* to enhance its expression (Fig. 9.4e) (Lim et al. 2013). SOMNUS is a protein which inhibits seed germination, and DELLAs/ABI3/ABI5 interactions result in germination repression. These overall observations imply that DELLA-TF interactions are the central event that enables the DELLA proteins to exert its repressive activity on GA signaling.

9.8 Semi-dwarf Breeding in Wheat

The green revolution in wheat sets a precedent for semi-dwarf breeding in rice and hence is indispensable in this chapter. Instrumental to the success of breeding shorter-stalked wheat is the semi-dwarf wheat variety, Norin 10. Wheat is a hexaploid and possesses chromosomes A, B, and D. Later studies identified two semidominant genes, *Rht1* (*Rht-B1b*) and *Rht2* (*Rht-D1b*), on chromosomes 4B and 4D, respectively, that are responsible for the semi-dwarf phenotype of Norin 10 (Peng et al. 1999; Hedden 2003). The wild-type *RHT1*/*RHT2* genes encode DELLA proteins which represses GA signaling (Peng et al. 1999; Alvey and Harberd 2005; Achard et al. 2006). Since *Rht-B1b* and *Rht-D1b* produce truncated proteins that lack the DELLA domain, the interaction between the GA/GID1 (GA receptor) complex and DELLA protein is blocked. This results in the accumulation of free DELLA proteins, which constitutively represses GA signaling and reduces plant height (Peng et al. 1999). Wheat has a hexaploid genome, so recessive mutations such as the *sd1* in rice cannot be used for wheat breeding. The selection and use of dominant mutants of GA signaling is a more reasonable tool for semi-dwarf breeding in wheat.

Norin 10 is a semi-dwarf variety selected by G. Inazuka and S. Asanuma from the progenies of crosses made between Turkey Red and the Japanese semi-dwarf variety Fultz-Daruma in 1935 in Japan. After World War II, Norin 10 was brought from Japan to the United States (Reitz and Salmon 1968). It was used as a genetic resource for wheat breeding in the United States, and in 1961, the semi-dwarf variety Gaines was generated by crossing Norin 10 with the variety Brevor. Gaines was dominantly cultivated in the Pacific Northwest and was responsible for the increased grain yield of wheat in the 1960s (Reitz and Salmon 1968). Thereafter, progenies of Norin 10/Brevor and Norin 10/Burt were sent to Mexico and used successfully in combination with Mexican traditional varieties at the International Maize and Wheat Improvement Center in Mexico (CIMMYT) to develop several semi-dwarf varieties including Pitic 62, Penjamo 62, and several others that also possess disease resistance (Chahal and Gosal 2006). These semi-dwarf varieties were distributed and cultivated in various countries including India, Pakistan, Turkey, and Nepal, and used as genetic resources for wheat breeding in Europe. Much of the improvement in wheat productivity can be attributed to the adoption of Norin 10 and its derivatives in crop production as well as in semi-dwarf breeding in wheat (Borojevic and Borojevic 2005).

9.9 Conclusion

The introduction of the semi-dwarf gene, *sd1*, to various rice varieties has markedly increased grain production. Many alleles of *sd1* have been used in independent breeding programs across many countries for decades. Even today, *sd1* is still

widely introgressed into existing, elite rice varieties, demonstrating the importance and usefulness of *sd1* in rice breeding. It is interesting to note that while a recessive mutation in *SD1*, GA biosynthetic gene, was instrumental in driving the green revolution in rice, a dominant mutation in *Rht*, a GA signal transduction gene, played the key role in the green revolution in wheat. These indicate that control of GA is important in cereal breeding for improved plant architecture.

Aside from breeding applications, identification and gene cloning from dwarf mutants had also been crucial in our understanding of the molecular mechanisms underlying phytohormone biosynthesis, signaling pathways, and interactions. Aside from dwarfism, no other trait in the history of rice research has greatly impacted rice production and advanced our fundamental understanding of one of the most important regulators of plant growth.

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Chapter 10
Genetic and Molecular Dissection of Flowering Time Control in Rice

Kazuki Matsubara and Masahiro Yano

Abstract Flowering time is one of the most important agronomic traits in rice (*Oryza sativa* L.) and is primarily controlled by quantitative trait loci (QTLs) that are associated with a photoperiodic response, particularly in short-day (SD) plants such as rice. Since the early twentieth century, rice breeders and researchers have been interested in clarifying the genetic control of flowering time because its modification is important for regional adaptation. The sequencing of the rice genome has facilitated genome-wide mapping of loci and gene cloning; thus, more progress has been made in elucidating the genetic control pathways of flowering. In this chapter, we provide an overview of the studies investigating rice flowering.

Keywords Flowering time · Genetic architecture · Genetic pathway · Photoperiod · QTL · Regional adaptation

10.1 Introduction

Flowering is the dramatic transition from the vegetative phase to reproductive development and is predominantly regulated by genetic control pathways that integrate internal and external signals. The ability of plant species to initiate flowering at the most favourable time for reproduction depends primarily on their accurate measurement of seasonal changes in day length and temperature (Thomas and Vince-Pure 1997; Song et al. 2015).

The flowering time (often termed the heading date) is important for regional adaptability and is easy to observe; therefore, its variations among rice varieties (*Oryza sativa* L.) have been known for a long time. Studies investigating the inheritance of rice flowering time date back to the 1910s. Hoshino (1915) suggested that multiple loci were involved in the inheritance of the flowering time based on

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the segregation patterns in progeny from experimental crosses between early- and late-flowering varieties. Since the 1920s, due to the development of the chromosome theory of inheritance and the discovery of genetic linkage, the linkage relationship between the flowering time genes and known loci (for other phenotypic traits) has been investigated (Chao 1928; Jodon 1940; Chandraratna 1953).

After the discovery of photoperiodism in plants (i.e. the response of an organism to the relative length of the day and night) by Garner and Allard (1920, 1923), many researchers have measured the flowering responses in rice varieties by day length. These studies revealed that rice is a facultative short-day (SD) plant as follows: its flowering is promoted under SD conditions, and the difference in the photoperiod response among rice varieties results in extensive variations in the flowering time (Vergara and Chang 1985).

The development of DNA markers in the 1990s dramatically enhanced the determination of the chromosomal location of genes/quantitative trait loci (QTLs) that are involved in the flowering time, and after decoding the genome sequences, map-based cloning strategies have facilitated the molecular cloning of these genes (Hori et al. 2016) (see also Chaps. 8 and 9 and those from Chaps. 11, 12, 13, 14, 15, 16, and 17).

In this chapter, we summarise the findings of many studies investigating rice flowering time using forward- and reverse-genetic approaches based on genomic information from rice. The studies performed over the last two decades have clearly shown that the use of a combination of these approaches has enhanced our understanding of the genetic control pathways of flowering in rice.

10.2 Mapping of the QTLs Responsible for Flowering Time

In the 1990s, the development of DNA markers allowed researchers to clarify the number and effects of the genes underlying the flowering time using QTL analyses (Li et al. 1995; Xiao et al. 1996; Yano et al. 1997). In particular, using several types of progeny derived from a single cross between the rice varieties 'Nipponbare' (ssp. *japonica*) and 'Kasalath' (ssp. *indica*), our group detected several QTLs responsible for the flowering time. Five of these QTLs (*Hd1–Hd5*) were mapped by performing a QTL analysis of an F_2 population (Yano et al. 1997), and *Hd7*, *Hd8*, and *Hd11* were detected in the BC_1F_5 lines (Lin et al. 1998). Other loci were detected only when advanced backcross progeny, such as BC_3F_2 or BC_4F_2, was used (Yamamoto et al. 2000; Lin et al. 2002). The results of these QTL mapping studies are summarised by Yano et al. (2001). Since then, many research groups have detected the QTLs for flowering time using different cross combinations. More comprehensive genetic analyses revealed that more than 100 loci, including major and minor effects, might be involved in flowering time control (Hori et al. 2016).

10.3 Molecular Cloning of Flowering Time Genes

Efforts detecting QTL above mentioned have led to the map-based cloning of the genes responsible for flowering time and improved our understanding of the function of these genes at the molecular level and the genetic pathways controlling flowering in rice.

Hd1 was the first rice flowering time gene cloned using natural variation (Table 10.1; Yano et al. 2000). Using more than 9000 recombinants, we defined the *Hd1* region within 12 kb on chromosome 6. This region contained one candidate gene with a high similarity to *Arabidopsis CONSTANS* (*CO*). Comparison of the candidate gene in 'Nipponbare' and 'Kasalath' revealed many sequence variations, including a 36-bp insertion and a 33-bp deletion (in exon 1) and a 2-bp deletion

Table 10.1 Molecularly cloned genes underlying the flowering time of rice

Gene symbol	Locus ID	Effect on flowering	Natural variation	Description
Hd1	Os06g0275000	SD promotion/LD repression	Known	Zinc finger protein
RFT1	Os06g0157500	LD promotion	Known	Florigen
OsTrx1	Os09g0134500	LD repression	Unknown	Trithorax group protein
OsMADS50/DTH3	Os03g0122600	SD/LD promotion	Known	MIKC-type MADS-box protein
OsMADS56	Os10g0536100	LD promotion	Unknown	Similar to MADS-box transcription factor 56
OsMADS15	Os07g0108900	SD/LD promotion	Unknown	Similar to MADS-box transcription factor 15
Hd3a	Os06g0157700	SD promotion	Known	Florigen
Ehd1	Os10g0463400	SD/LD promotion	Known	B-type response regulator
Ehd2	Os10g0419200	SD/LD promotion	Unknown	Cys2/His2-type zinc finger transcription factor
Ehd3	Os08g0105000	LD promotion	Unknown	Homeodomain (PHD) transcriptional regulator
Ehd4	Os03g0112700	SD/LD promotion	Known	Zinc finger CCCH domain-containing protein
OsCOL4	Os02g0610500	SD/LD repression	Unknown	CO-like protein containing two B-box zinc finger domains and one CCT domain
OsCOL10	Os03g0711100	SD/LD repression	Unknown	Member of the CONSTANS-like (COL) family
Hd6	Os03g0762000	LD repression	Known	Similar to protein kinase CK2, alpha subunit

(continued)

Table 10.1 (continued)

Gene symbol	Locus ID	Effect on flowering	Natural variation	Description
Ghd7	Os07g0261200	LD repression	Known	CCT (CONSTANS, CONSTANS-like, and timing of chlorophyll A/B binding1) domain protein
DTH2	Os02g0724000	LD promotion	Known	CONSTANS-like protein
Se14	Os03g0151300		Unknown	Jumonji C domain-containing protein
OsMADS51	Os01g0922800	SD/LD promotion	Unknown	MADS-box transcription factor
DTH8	Os08g0174500	LD repression	Known	Putative HAP3 subunit of CCAAT box-binding transcription factor
OsLFL1	Os01g0713600	LD repression	Unknown	Transcriptional factor B3 family protein
OsPRR37	Os07g0695100	LD repression	Known	Pseudo-response regulator
Hd18	Os08g0143400	SD/LD promotion	Known	SWIRM and amine oxidase domain-containing protein
OsVIL2	Os02g0152500	LD promotion	Unknown	Chromatin remodelling factor
Se13	Os01g0949400		Unknown	Similar to Phytochromobilin synthase precursor
Hd17	Os06g0142600	SD/LD promotion	Known	Homolog of *Arabidopsis* early flowering 3 protein
Hd16	Os03g0793500	LD repression	Known	Casein kinase I
OsFD	Os07g0658400	SD/LD promotion	Unknown	Ferredoxin-dependent glutamate synthase
GF14C	Os08g0430500	SD/LD promotion	Unknown	14-3-3 protein
OsGI	Os01g0182600	SD/LD promotion	Unknown	GIGANTEA protein

Locus ID was based on the Rice Annotation Project (http://rapdb.dna.affrc.go.jp/)

(in exon 2) in 'Kasalath'. A small genomic fragment of 'Nipponbare' containing the *Hd1* candidate gene was transferred into the near isogenic line of 'Nipponbare' carrying *Hd1* from 'Kasalath' and was found to promote flowering under SD conditions. These results clearly indicated that the candidate gene homologous to *Arabidopsis CO* was *Hd1*.

At least 14 flowering time QTLs have been isolated using map-based cloning strategies assessing natural variation (Yano et al. 2000; Takahashi et al. 2001; Kojima et al. 2002; Doi et al. 2004; Xue et al. 2008; Wei et al. 2010; Bian et al. 2011; Matsubara et al. 2012; Gao et al. 2013; Hori et al. 2013; Koo et al. 2013;

Ogiso-Tanaka et al. 2013; Wu et al. 2013; Shibaya et al. 2016). Rice mutants have also been used to isolate flowering time genes and investigate their functions (Izawa et al. 2000; Lee et al. 2004; Matsubara et al. 2008, 2011; Saito et al. 2012; Dai and Xue 2010; Yang et al. 2013; Yokoo et al. 2014; Yoshitake et al. 2015). Other flowering time genes have been identified, and their functions have been investigated using forward- and reverse-genetic approaches, such as overexpression or knockdown of a target gene. For example, the functions of *RFT1* and *OsTrx1* were revealed by knocking down these genes, whereas the functions of *OsMADS50*, *OsMADS56*, and *OsMADS15* were verified by overexpressing these genes (Komiya et al. 2008; Ryu et al. 2009; Lu et al. 2012; Choi et al. 2014).

10.4 Genetic Pathways Controlling Flowering Revealed by Molecular Cloning

Under SD conditions, rice flowering is promoted by the expression of *Hd3a*, which is activated by Hd1 and Ehd1 (Table 10.1; Yano et al. 2000; Kojima et al. 2002; Doi et al. 2004; Tamaki et al. 2007) (Fig. 10.1). Hd3a acts as a mobile flowering signal (florigen) (Tamaki et al. 2007). The expression of *Ehd1* is upregulated by *DTH3*, *Ehd2*, *Ehd3*, *Ehd4*, and *OsMADS51* (Kim et al. 2007; Matsubara et al. 2008, 2011; Bian et al. 2011; Gao et al. 2013) and downregulated by *OsCOL4* and *OsCOL10* (Lee et al. 2010; Tan et al. 2016).

The transcriptional activation of *Hd3a* is lower under long-day (LD) conditions than under SD conditions; consequently, flowering is suppressed (Fig. 10.1). Although Hd1 activates the expression of *Hd3a* under SD conditions, Hd1 represses the expression of *Hd3a* under LD conditions (Fig. 10.1). This functional conversion of Hd1 is caused by phytochrome-mediated signalling (Hayama and Coupland 2004; Izawa 2007). The Hd1 repressor function under LD conditions is enhanced by the kinase activity of *Hd6* and is mediated by unknown genes (Takahashi et al. 2001; Ogiso et al. 2010). In addition to *Hd1*, *Ghd7* also represses the expression of *Hd3a* by repressing *Ehd1* under LD conditions (Xue et al. 2008). Based on genetic analyses, it was originally believed that rice photoperiodic flowering is controlled by the following two independent signalling pathways: the *Hd1–Hd3a* pathway, which is evolutionarily related to the *Arabidopsis CO–FT* pathway, and the *Ghd7-Ehd1–Hd3a* pathway, which has no *Arabidopsis* counterpart (Doi et al. 2004; Xue et al. 2008). However, a physical interaction was recently demonstrated between Hd1 and Ghd7 in vivo (Nemoto et al. 2016). The protein complex of Hd1 and Ghd7 specifically binds to a *cis*-regulatory region in *Ehd1* and represses its expression, suggesting that the two pathways are integrated into *Ehd1* and repress flowering under LD conditions (Fig. 10.1).

Due to the progress in our understanding of the core pathways, many genes underlying rice flowering under LD conditions have been discovered during the last decade (Fig. 10.1). *RFT1*, which is located within 11.5 kb of *Hd3a*, is an *Hd3a*

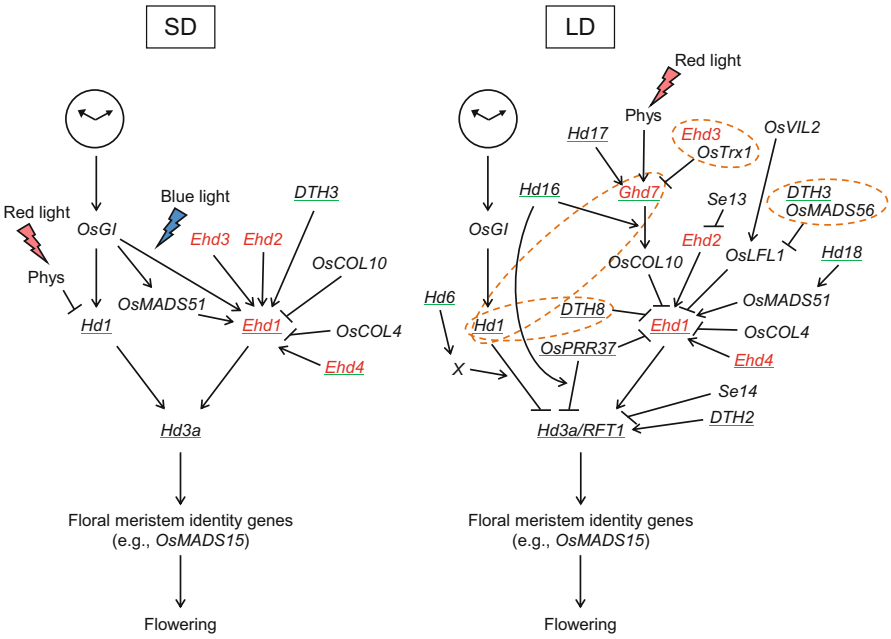

Fig. 10.1 A schematic representation of the genetic pathways controlling flowering in rice. The clocks at the *top* show the circadian clock. Genes with no obvious *Arabidopsis* counterparts are shown in *red*. *Orange* ovals show physical interactions between genes. Genes with a natural allelic variation are underlined. *X* indicates an unknown gene. SD short-day conditions, LD long-day conditions. Arrows upregulation, bars downregulation. Phys phytochromes

paralog (Kojima et al. 2002; Komiya et al. 2008). The expression of *RFT1* increases under LD conditions, and RFT1 moves from the leaves to the shoot apical meristem, indicating that the control of the flowering time in rice involves two florigen genes, *Hd3a* and *RFT1*, under LD conditions (Komiya et al. 2008, 2009). The expression of *RFT1* is promoted by *Ehd1* and *DTH2* but is repressed by *Se14* (Doi et al. 2004; Wu et al. 2013; Yokoo et al. 2014). The expression of *Ehd1* is induced by *DTH3*, *OsMADS51*, *OsMADS56*, *Ehd2*, and *Ehd4* (Kim et al. 2007; Matsubara et al. 2008; Ryu et al. 2009; Bian et al. 2011; Gao et al. 2013) but is repressed by *DTH8*, *OsCOL4*, *OsCOL10*, *OsLFL1*, and *OsPRR37* (Peng et al. 2008; Lee et al. 2010; Wei et al. 2010; Yan et al. 2011; Gao et al. 2014; Tan et al. 2016). Recently, DTH8 has been reported to form a complex with Hd1 to control flowering (Chen et al. 2014; Zhu et al. 2017). DTH3 and OsMADS56 form a complex that regulates *Ehd1* (Ryu et al. 2009). *OsMADS51* is upregulated by *Hd18* and induces the expression of *Ehd1* (Kim et al. 2007; Shibaya et al. 2016). OsLFL1 is induced by *OsVIL2* and has been proposed to downregulate the expression of *Ehd1* (Peng et al. 2008; Yang et al. 2013). *Ehd2* is downregulated by *Se13* and induces the expression of *Ehd1* (Matsubara et al. 2008; Yoshitake et al. 2015). *OsCOL4* is a constitutive

repressor upstream of *Ehd1* (Lee et al. 2010). *OsCOL10* downregulates the expression of *Ehd1* and is upregulated by *Ghd7* (Tan et al. 2016). The expression of *Ghd7* is induced by *Hd17* and is repressed by the Ehd3 and OsTrx1 complex (Choi et al. 2014). Ghd7 activity is increased by phosphorylation by Hd16 (Hori et al. 2013). Hd16 also phosphorylates *OsPRR37*, which represses the expression of *Hd3a* either directly or through *Ehd1* (Hori et al. 2013; Koo et al. 2013; Gao et al. 2014).

Thus, under LD conditions, most flowering time gene signals (by both repressors and promoters) are transmitted to rice florigen genes through *Ehd1* in flowering rice (Fig. 10.1).

The shared regulation by flowering time genes downstream of *Hd3a* and *RFT1* is an underlying mechanism under both SD and LD conditions (Fig. 10.1). Hd3a interacts with GF14C, and then the Hd3a-GF14C complex interacts with OsFD (Taoka et al. 2011; Tsuji et al. 2013). The resultant protein complex induces the expression of floral meristem identity genes (e.g. *OsMADS15*) to initiate the floral transition in the shoot apex.

An additional description of the genes described above is provided in Table 10.1.

10.5 Circadian Clock Genes Control the Expression of Flowering Time Genes

The expression of many rice flowering time genes depends on the day length (Izawa 2007; Itoh et al. 2010). For example, *Hd3a* and *Ehd1* are expressed in the morning under SD conditions, whereas *Ghd7* is expressed in the morning under LD conditions. The expression of *OsGI* shows daily circadian oscillations with a peak at the end of the light period, and the expression of *OsGI* is regulated by the circadian clock and activates the expression of *Hd1* (Table 10.1; Hayama et al. 2002, 2003) (Fig. 10.1). OsGI also activates the expression of *Ehd1* either directly or via *OsMADS51* (Kim et al. 2007; Itoh et al. 2010). The expression levels of certain flowering time genes are regulated by the circadian gating of light responses through phytochromes (red-light receptors) and cryptochromes (blue-light receptors) (Itoh et al. 2010). The expression of *Ehd1* is induced by blue light in an *OsGI*-dependent manner regardless of day length; however, the expression is repressed by *Ghd7* under LD conditions. The expression of *Ghd7* is induced by phytochrome signalling, and the sensitivity to red light is gated at the beginning of the light period under LD conditions.

Recently, Matsuzaki et al. (2015) developed a statistical model of the expression of multiple genes with phase setting by sunlight and the circadian clock under field conditions. The integration of the expression patterns of individual flowering time genes can accurately estimate the internal biological time determined by both the circadian clock and the actual physical time of day.

To date, the control of flowering by the circadian clock in rice remains largely unknown compared to that in *Arabidopsis* likely because experiments in the laboratory are difficult to perform due to its large plant size. However, field experiments, such as those performed by Matsuzaki et al. (2015), will provide a better understanding of the role of the circadian clock in rice flowering.

10.6 Genetic Architecture of the Natural Variations in Flowering Time

To clarify the natural allelic variations in flowering time, we carried out QTL analyses in 12 F_2 populations derived from crosses of 'Koshihikari' (ssp. *japonica*), which is an elite Japanese variety that is commonly used as a parental line, with varieties originating in various regions in Asia (Ebana et al. 2011; Shibaya et al. 2011). A limited number of loci with large effects that corresponded to *Hd1*, *Hd2*, *Hd6*, *RFT1*, *Ghd7*, *DTH8*, and *Hd16* accounted for some varietal differences, but additional QTLs are likely to be involved in the flowering variation in these populations.

To detect QTLs with small effects, we analysed advanced backcross progeny derived from each cross combination by Ebana et al. (2011) and Shibaya et al. (2011) and detected a total of 255 QTLs widely distributed across the genome (Hori et al. 2015). We detected 128 QTLs with a relatively large effect, which corresponded to the genomic positions of previously detected flowering time genes, such as *Hd1*, *Hd2*, *Hd6*, *RFT1*, *Ghd7*, *DTH8*, and *Hd16*. The sequence analyses revealed that the chromosomal positions of the large-effect QTLs mainly corresponded to those of different alleles of the flowering time genes in 12 rice varieties. The other 127 QTLs were detected in chromosomal regions other than those of the flowering time genes and had relatively small effects. These results indicate that much of the variation in the flowering time can be explained by combinations of alleles in large- and small-effect QTLs.

Genome-wide association studies have also supported the hypothesis that allelic variations at multiple QTLs play an important role in the differences in the flowering time among rice varieties (Zhao et al. 2011; Huang et al. 2012; Yano et al. 2016). Zhao et al. (2011) detected ten genomic regions that were significantly associated with the flowering time variation, although only *Hd1* was detected as a major QTL. These genomic regions explained less than 50% of the flowering time variation. Huang et al. (2012) found 14 significant genomic regions: 5 regions surrounding *Hd1*, *Ghd7*, *RCN1*, *OsGI*, and *Hd3a* and 9 newly discovered regions. The detected regions explained 36% of the flowering time variation. More recently, a genome-wide association study revealed that two novel QTLs on chromosomes 1 and 11 contributed to the flowering time variation in *japonica* rice varieties (Yano et al. 2016). The above-mentioned studies suggest that QTLs that have not yet been

discovered are associated with the natural variation in the flowering time in rice varieties.

10.7 Regional Adaptation Based on Allelic Differences in Flowering Time Genes

Early flowering conferred by deficient alleles in the flowering time genes is important for expanding the range of rice cultivation to high latitudes (LD conditions) (Izawa 2007; Shrestha et al. 2014), where early heading and maturity are required for seed production. A sequence analysis of the known flowering time genes, including *Hd1*, *Ghd7*, *DTH8*, *Hd16*, *OsPRR37*, *DTH2*, and *Ehd4*, indicated that allelic differences contribute to regional adaptation (Takahashi et al. 2009; Naranjo et al. 2014; Gómez-Ariza et al. 2015; Zheng et al. 2015; Goretti et al. 2017). The functional alleles of *Hd1* are associated with late flowering, and its non-functional alleles are associated with early flowering under natural day-length conditions; the geographical distribution of the *Hd1* alleles suggests that favourable alleles have been selected by breeders to enhance rice productivity and adaptability in each region (Takahashi et al. 2009; Fujino et al. 2010; Ebana et al. 2011; Takahashi and Shimamoto 2011; Naranjo et al. 2014; Gómez-Ariza et al. 2015; Goretti et al. 2017). The deficient or weak alleles of *Ghd7*, *DTH8*, *DTH2*, *Hd16*, and *OsPRR37* are distributed in cultivation areas at high latitudes (Xue et al. 2008; Wei et al. 2010; Fujino et al. 2013; Hori et al. 2013; Koo et al. 2013; Wu et al. 2013; Kwon et al. 2014; Goretti et al. 2017), suggesting that these alleles must be involved in the expansion of rice cultivation areas.

10.8 Conclusions and Perspectives

During the last two decades, tremendous progress in genome sequencing has improved our understanding of the genetic and molecular mechanisms that control the flowering time in rice. For example, *Ehd1* is an important integrator in genetic control pathway, the putative homolog has found in sorghum (SD plant); on the other hand, it has not been found in *Arabidopsis* and wheat (LD plants) (Brambilla et al. 2017). Such a finding in rice provides valuable suggestion about the genetic control of flowering time in sorghum; additionally, it plays an important role in understanding of the diversity and evolution of flowering time control in plants.

This progress was due not only to genomic approaches, such as QTL analyses and map-based cloning, but also to the large number of rice accessions (including wild relatives) and genetic mapping populations derived from artificial crosses. Most genes with major effects on flowering time have already been identified

during the last 20 years. Although some genes with minor effects have also been analysed, additional new QTLs with minor effects need to be further examined. In fact, genome-wide association studies could be an effective method for detecting new chromosomal regions (QTLs) responsible for the flowering time (Yano et al. 2016). The verification of the allelic effects of these QTLs with minor effects must be performed using experimental populations derived from single crosses. This approach will lead a more in-depth understanding of the genetic control of flowering time in rice. Information derived from these analyses has also been applied to the modulation of the flowering time of cultivars for regional adaptation and cropping system (Takeuchi et al. 2006; Takeuchi 2011; Hori et al. 2016).

Recently, genome-wide prediction models of the flowering time in rice have been tested and demonstrated high prediction accuracy by adding environmental variables (Nakagawa et al. 2005; Onogi et al. 2016; Spindel et al. 2016). Furthermore, it appears that these models can predict the flowering time using genome-wide genotypes and trait values in various types of populations, such as experimental biparental populations or an array of varieties (Spindel et al. 2016). Further progress using these approaches will enhance the fine-tuning of flowering time in rice breeding programmes.

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Chapter 11
Gene Network of Grain Size and Number in Rice

Qian Qian

Abstract Grain size and number are two key components determining rice yield. As both phenotypes are characterized by quantitative traits, grain size and number are predominantly and tightly controlled by genetic factors. Several quantitative trait loci (QTLs) responsible for grain size and number have been molecularly identified and characterized. These QTLs may act in independent genetic pathways and, along with other identified genes for grain size and number, are mainly involved in the signaling pathways mediated by the ubiquitin-proteasome pathway, G-protein signaling, phytohormones, and transcriptional regulatory factors. Since many of these QTLs and genes were identified from modern elite varieties, they have been strongly selected for enhanced rice productivity in the breeding program. These findings have paved new ways for understanding the molecular basis of grain size and number and enable us to develop the desirable crops with high yield by precise design.

Keywords Grain size · Grain number · Quantitative trait loci · Phytohormone · Transcription factor · Rice

11.1 Introduction

Rice, one of the most important crops, supplies the food for more than half of the global population. Grain yield of rice is mainly determined by the number of panicles, the number of grains per panicle, and grain weight (Sakamoto and Matsuoka 2008; Xing and Zhang 2010). These agronomically important traits have been extensively studied, and great progress has been made in the past decades (Huang et al. 2013; Zuo and Li 2014; Miura et al. 2011; Wang and Li 2008, 2011; Li and Li 2016; Yu et al. 2013; Zhou et al. 2013). Grain size, the major factor

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affecting grain weight and shape, is determined by grain length (GL), grain width (GW), and grain thickness (GT). Grain shape is also an important appearance quality trait of rice due to the preference differences of the consumers from different habitat areas of the world. In addition, grain size usually becomes the target traits selected during domestication and breeding, thus providing an excellent model for evolution studies (Meyer and Purugganan 2013; Moles et al. 2005; Sweeney and McCouch 2007). Grain number, another important agronomic trait, is controlled by both genetic and environmental factors of rice. This trait is also associated with plant architecture in rice and indirectly regulates grain size (Huang et al. 2009). Recently, some quantitative trait loci (QTLs) related to grain size and number have been cloned. Characterization of these genes has shed great light on the molecular mechanisms of the important agronomic traits which paved a new way for generation of the new super rice (Huang et al. 2013; Miura et al. 2011; Wang and Li 2011; Xing and Zhang 2010; Yu et al. 2013; Zuo and Li 2014).

In this chapter, we focus on the natural variation in the genes/QTLs related to grain size and number in rice. Especially, we highlight the emerging molecular mechanisms underlying newly identified regulators of seed size and number in rice (see also Chap. 12).

11.2 Genes for Grain Size and Number

Using distinct mapping population, tremendous genes/QTLs for grain yield traits have been detected in rice. Among them, several major QTLs for grain size and number have been cloned, and their regulatory roles in determining grain size and number have also been explored (Table 11.1).

11.2.1 GRAIN SIZE 2

GS2 (*GRAIN SIZE 2*), also named *GL2* (*GRAIN LENGTH 2*) or *PT2* (*PANICLE TRAITS 2*), is a rare allele affecting both grain length and width in rice. *GS2* encodes a transcriptional regulator named Growth-Regulating Factor 4 (OsGRF4) and is targeted by a microRNA, OsmiR396. A 2-bp substitution mutation in *GS2* perturbs the binding site of *GS2*/*OsGRF4* by OsmiR396, resulting in elevated expression of *GS2*/*OsGRF4*. This gain-of-function mutation leads to larger cells and increased numbers of cells in the seed, which thus enhances grain weight and yield (Hu et al. 2015; Che et al. 2015; Duan et al. 2015; Sun et al. 2016). The increased expression of *GS2*/*OsGRF4* activates brassinosteroid responses by upregulating a large number of brassinosteroid (BR)-induced genes. GSK2, a central negative regulator of rice brassinosteroid signaling, was found to directly interact with and repress the transcription activation activity of GS2/OsGRF4 to mediate the specific regulation of grain length (Che et al. 2015). Besides controlling

Table 11.1 List of major quantitative trait loci (QTLs) controlling grain size and number

QTLs	Locus ID	Traits	Annotation	References
GS2	Os02g0701300	Grain length and width	Transcription factor	Che et al. (2015), Duan et al. (2015), Hu et al. (2015), and Sun et al. (2016)
GS3	Os03g0407400	Grain length and width	G-protein γ-subunit	Fan et al. (2006), Mao et al. (2010), and Takano-Kai et al. (2013)
GS5	Os05g0158500	Grain width and filling	Serine carboxypeptidase	Li et al. (2011a)
GS6	Os06g0127800	Grain width and weight	GAI-RGA-SCR (GRAS) family protein	Sun et al. (2013)
qGL3	Os03g0646900	Grain width, length, and weight	Protein phosphatase	Hu et al. (2012), Qi et al. (2012), and Zhang et al. (2012)
GW2	Os02g0244100	Grain width and weight	RING-type E3 ligase	Song et al. (2007)
GW5/ *qSW5*/ *GSE5*	Os05g0187500	Grain width and weight	Calmodulin-binding protein	Shomura et al. (2008), Weng et al. (2008), Duan et al. (2017), and Liu et al. (2017)
GW7/ *GL7*/ *SLG7*	Os07g0603300	Grain length and width	TONNEAU1-recruiting motif protein	Wang et al. (2015a, b), and Zhou et al. (2015)
GLW7	Os07g0505200	Grain length and weight	Squamosa promoter-binding-like protein	Si et al. (2016)
GW6a	Os06g0650300	Grain weight	Histone H4 acetyltransferase	Song et al. (2015)
GW8	Os08g0531600	Grain width	Squamosa promoter-binding-like protein	Wang et al. (2012)
TGW6	Os06g0623700	Grain weight	IAA-glucose hydrolase	Ishimaru et al. (2013)
Gn1a	Os01g0197700	Grain number	Cytokinin dehydrogenase	Ashikari et al. (2005)
IPA1/ *WFP*	Os08g0509600	Grain number	Squamosa promoter-binding-like protein	Jiao et al. (2010), Miura et al. (2010), and Lu et al. (2013)

grain size, *GS2*/*OsGRF4* (*PT2*/*OsGRF4*) also regulates rice panicle development through affecting the expression of two cytokinin dehydrogenase precursor genes (*CKX1* and *CKX5*) to increased cytokinin (CK) levels (Sun et al. 2016). These results demonstrated that BR and CK signaling pathway plays a pivotal role in rice grain and panicle development.

11.2.2 GRAIN SIZE 3

GS3 (*GRAIN SIZE 3*) was the first gene molecularly characterized to control both grain weight and grain length, with minor effects on grain width and thickness. *GS3* was identified as a major QTL from a population derived from a cross between a large-grain cultivar (Minghui 63) and a small-grain one (Chuan 7) (Fan et al. 2006). By map-based cloning, *GS3* was isolated to encode a putative transmembrane protein containing a plant-specific organ size regulation (OSR) 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 (Fan et al. 2006; Mao et al. 2010). Four *GS3* alleles were detected in 82 accessions. Zhenshan 97 (Haplotype-1) and Nipponbare (Haplotype-2) exhibiting intermediate grain size contain all the predicted domains. All 11 long-grain cultivars like Minghui 63 (Haplotype-3) carry a nonsense mutation that causes the formation of a polypeptide lacking all major functional domains. Represented by Chuan 7 - (Haplotype-4), several variants, with various mutations in exon 5 of *GS3* resulting in truncated proteins lacking the TNFR/NGFR and VWFC domains, show the strongest phenotype in inhibiting grain length (Mao et al. 2010; Takano-Kai et al. 2013). These studies demonstrated that *GS3* is a negative regulator of grain size, and the OSR domain functions as a negative regulatory motif whose activity is repressed by the TNFR/NGFR and VWFC domains (Fan et al. 2006; Mao et al. 2010; Takano-Kai et al. 2013). At the cellular level, *GS3* alters seed length by regulating the cell number in the upper epidermis of the glume, with minor effects on the cell size, indicating an important role of *GS3* in regulating cell division (Takano-Kai et al. 2013).

11.2.3 GRAIN SIZE 5

GS5 (*GRAIN SIZE 5*) was detected from a double haploid (DH) population derived from a cross between Zhenshan 97 (wide grain) and H94 (slender grain). *GS5* controls grain size by regulating grain width, filling, and weight. *GS5* encodes a putative serine carboxypeptidase and acts as a positive regulator of a subset of the G1-to-S transition genes of the cell cycle. At the cell level, *GS5* promotes cell division and, to a lesser extent, cell elongation of the palea and lemma. Sequencing of the promoter region in 51 rice accessions revealed that the *GS5*-increased grain size is caused by polymorphisms in the *GS5* promoter, which is associated with the different expression levels of *GS5* (Li et al. 2011a).

11.2.4 GRAIN SIZE 6

GS6 (*GRAIN SIZE 6*) encodes a GRAS family protein and negatively regulates grain size in rice. Loss-of-function of *GS6* increased the grain width and weight. Comparison of the *GS6* CDS regions in 90 rice accessions revealed three *GS6* alleles. Most *japonica* varieties (95%) harbor the type I haplotype, and 62.9% of *indica* varieties harbor the type II haplotype. Association analysis showed that the type I haplotype tends to increase the width and weight of grains more than either of the type II or type III haplotypes. Further investigation of genetic diversity and the evolutionary mechanisms of *GS6* showed that the *GS6* gene was strongly selected in *japonica* cultivars (Sun et al. 2013).

11.2.5 GRAIN LENGTH 3

Using different mapping population, a major QTL named *qGL3*/*qGL3.1* (*GRAIN LENGTH 3*) was identified by three groups (Hu et al. 2012; Qi et al. 2012; Zhang et al. 2012). *qGL3*/*qGL3.1* encodes a putative serine/threonine protein phosphatase containing a Kelch-like repeat domain (OsPPKL1) and affects remarkably the traits related to rice grain (grain length, width, thickness, weight, and filling). A beneficial allele was attributed to a mutation, Asp-to-Glu transition at residue 364 in the conserved AVLDT motif of the second Kelch domain, which caused cultivars with this allele to produce heavier grains, indicating that Asp^{364} plays a critical role for the phosphatase activity of qGL3/qGL3.1 (Hu et al. 2012; Qi et al. 2012; Zhang et al. 2012). Accordingly, the wild-type qGL3/qGL3.1 protein shows higher phosphatase activity than the mutated proteins on its substrate, Cyclin-T1;3, and the knockdown of the *Cyclin-T1*;*3* expression resulted in shorter grains. The reduced phosphatase activity may alter the progression of the cell cycle, thereby causing the increased cell number in the outer glume and, consequently, longer grains (Qi et al. 2012; Zhang et al. 2012).

The rice genome has two other *qGL3*/*qGL3.1* homologs, *OsPPKL2* and *OsPPKL3*. Transgenic studies showed that *OsPPKL1* and *OsPPKL3* function as negative regulators of grain length, whereas *OsPPKL2* as a positive regulator. The Kelch domains are essential for the OsPPKL1 biological function. Field trials showed that the application of the *qgl3* allele could significantly increase grain yield in both inbred and hybrid rice varieties (Zhang et al. 2012).

11.2.6 GRAIN WIDTH 2

The *GW2* (*GRAIN WIDTH 2*) was detected in an F_2 population derived from a cross between a *japonica* variety WY3 (large grain) and an *indica* variety Fengaizhan-1

(FAZ1, small grain). Using positional cloning approach, *GW2* was isolated to encode a previously unknown RING-type protein with E3 ubiquitin ligase activity, which is known to function in the degradation by the ubiquitin-proteasome pathway. Disruption of *GW2* function (*gw2*) increased cell numbers at the outer parenchyma cell layer, resulting in a larger (wider) spikelet hull. *gw2* also accelerated the grain milk filling rate, resulting in enhanced grain width, weight, and yield. Although endosperm cells of NIL(FAZ-*gw2*) were larger than those of FAZ1, there was no significant difference in endosperm cell number, suggesting that the increase in NIL(FAZ-*gw2*) endosperm size is mainly due to the larger endosperm cells, and that *GW2* regulates development of the spikelet and endosperm by distinctive mechanisms. Taken together, *GW2* has a negative role in regulating grain size and weight (Song et al. 2007).

11.2.7 GRAIN WIDTH 5/SEED WIDTH 5

GW5/*qSW5* (*GRAIN WIDTH 5*/*SEED WIDTH 5*, also called *GSE5*) was independently identified as a major QTL responsible for grain width and weight in different recombinant inbred lines (RILs) generated from crosses between Asominori/Nipponbare (wide grains) and IR24/Kasalath (slender grains) (Shomura et al. 2008; Weng et al. 2008). *GW5*/*qSW5* was reported to be closely associated with a 1212-bp DNA deletion fragment causing decreased *GW5* expression and increased grain width (Shomura et al. 2008; Weng et al. 2008; Duan et al. 2017). In previous studies, *GW5*/*qSW5* was described to encode a novel nuclear protein that is localized in the nucleus and physically interacts with polyubiquitin (Shomura et al. 2008; Weng et al. 2008). However, two latest papers reported that its neighboring gene is actually the *GW5*/*qSW5* which encodes a calmodulin-binding protein, and GW5/qSW5 physically interacts with the rice calmodulin OsCaM1-1 (Duan et al. 2017; Liu et al. 2017). Knockout of *GW5*/*qSW5* by a CRISPR/Cas9 strategy produced a wider grain, while elevated expression level of the gene generated a narrower grain, suggesting that *GW5*/*qSW5* exerts a negative effect on grain width (Duan et al. 2017; Liu et al. 2017). In addition to the 1212-bp deletion that is present mainly in *japonica* varieties, a 950-bp deletion in *GW5*/*qSW5* that prevails in *indica* varieties is also closely linked to grain width alteration. Furthermore, the different *GW5*/*qSW5* alleles identified in cultivated rice could have originated from different wild accessions (Duan et al. 2017).

11.2.8 GRAIN WIDTH 7/GRAIN LENGTH 7

Using different mapping populations, *GW7*/*GL7* (*GRAIN WIDTH 7*/*GRAIN LENGTH 7*, also named *SLG7*) was identified by three independent studies (Wang et al. 2015a, b; Zhou et al. 2015). *GW7*/*GL7* encodes a TONNEAU1-

recruiting motif protein with similarity to C-terminal motifs of the human centrosomal protein CAP350. Upregulation of *GW7*/*GL7* expression was correlated with the production of more slender grains, resulting from increased cell division in the longitudinal direction and decreased cell division in the transverse direction. Therefore, *GW7*/*GL7* seems to regulate grain shape by changing cell division patterns (Wang et al. 2015a). Interestingly, it was found that tandem duplication of a 17.1-kb segment at the *GW7*/*GL7* locus leads to the higher expression of *GW7*/*GL7* and downregulation of its nearby negative regulator. Allelic variants of *GW7*/*GL7* and its negative regulator were selected in breeding due to an increase in grain length and improvement of grain appearance quality (Wang et al. 2015b).

11.2.9 GRAIN LENGTH AND WEIGHT 7

GLW7 (*GRAIN LENGTH AND WEIGHT 7*), a major QTL that controls grain length and weight, was identified by GWAS analysis of grain size (Si et al. 2016). *GLW7* encodes a SQUAMOSA promoter-binding protein-like 13 (OsSPL13), a member of the plant-specific SBP domain family of transcription factors. A tandem-repeat sequence in the 5′-UTR of *OsSPL13* alters its expression by affecting transcription and translation, and that high expression of *OsSPL13* is associated with large grains in tropical *japonica* rice. In addition to regulating panicle architecture, *OsSPL13* positively regulates cell size in the grain hull, resulting in enhanced rice grain length and yield (Si et al. 2016). At the cell level, *OsSPL13* regulates grain shape via control of the mechanisms determining cell size and predominantly regulates organ development by regulating the size that individual cells achieve during growth, rather than the number of cells comprising an organ (Si et al. 2016). Moreover, OsSPL13 binds to the promoter of the *SRS5* gene (SMALL AND ROUND SEED 5) and seems to positively regulate *SRS5* expression. *SRS5* encodes an α-tubulin subunit component of the microtubule cell growth machinery, thus providing a link between *OsSPL13* and cell growth regulation (Si et al. 2016).

11.2.10 GRAIN WEIGHT 6a

GW6a (*GRAIN WEIGHT 6a*) was detected in an F_2 population derived from a cross between CSSL29 (a chromosome segment substitution line from Nipponbare×Kasalath) and Nipponbare. *GW6a* encodes a new-type GNAT-like protein that harbors intrinsic histone acetyltransferase activity (OsglHAT1) (Song et al. 2015). Allelic variations were found in a 1.2-kb region upstream of the *OsglHAT1* gene body, which is consistent with its function as a positive regulator of the traits. Elevated *OsglHAT1* expression enhances grain weight and yield by enlarging spikelet hulls via increasing cell number and accelerating grain filling and increases global acetylation levels of histone H4. OsglHAT1 localizes to the

nucleus, where it likely functions through the regulation of transcription. Despite its positive agronomical effects on grain weight, yield, and plant biomass, the rare allele elevating *OsglHAT1* expression has so far escaped human selection. *GW6a*, as a chromatin modifier, is the first example of a QTL for a yield component trait (Song et al. 2015).

11.2.11 GRAIN WIDTH 8

GW8 (*GRAIN WIDTH 8*) is a positive regulator of cell proliferation and contributes to grain width and weight (Wang et al. 2012). *GW8* encodes a SQUAMOSA promoter-binding protein-like 16 (OsSPL16) that positively regulates the expression of several genes involved in the G1-to-S transition, a regulatory role similar to that of *GS5* (Li et al. 2011a; Wang et al. 2012). A higher expression level of *GW8* promotes cell division and grain filling, thereby increasing grain width and yield. Mutations in the *GW8* promoter, such as in Basmati varieties, cause the formation of more slender grains, which were preferred by breeders. Interestingly, a HJX74-NIL containing *gs3* and *gw8* shows an additive phenotype with a more slender grain than its parents, indicating that *GS3* and *GW8* genetically act in independent pathways. Practically, this fact also offers a strategy to simultaneously improve grain quality and yield in breeding (Wang et al. 2012).

11.2.12 THOUSAND-GRAIN WEIGHT 6

During endosperm development, grain filling is an important process which affects the grain size and weight. The timing of the transition from the syncytial to cellular phase is critical for endosperm development and eventually for rice grain size (Zhou et al. 2013). Using a mapping population generated between Nipponbare (wider grains) and Kasalath (slender grains), the QTL *TGW6* (*THOUSAND-GRAIN WEIGHT 6*) was identified by positional cloning, which revealed an important mechanism regulating grain filling during endosperm development (Ishimaru et al. 2013). The *TGW6* allele in Nipponbare encodes a novel protein with indole-3-acetic acid (IAA)-glucose hydrolase activity and positively regulates free IAA levels in grains. However, the Kasalath *tgw6* allele, carrying a premature stop codon caused by a frameshift mutation, had a remarkably reduced level of free IAA in *NILtgw6*. The increased IAA level in Nipponbare affects the timing of the transition from the syncytial to the cellular phase and limiting cell number and grain length, as evident by the observation that the number of endosperm cell layers in Nipponbare (*TGW6* allele) is significantly reduced compared with that of *NILtgw6*.

Moreover, loss-of-function of the Kasalath allele enhances grain weight through pleiotropic effects on source organs and leads to significant yield increases (Ishimaru et al. 2013).

11.2.13 *GRAIN NUMBER 1a*

Using a mapping population of 96 backcross inbred lines (BILs) derived from the cross between Habataki and Koshihikari, *Gn1*, the most effective QTL for increasing grain number from Habataki, was detected (Ashikari et al. 2005). By high-resolution mapping, two loci *Gn1a* (*GRAIN NUMBER 1a*) and *Gn1b* at the *Gn1* locus were identified, in which *Gn1a* was successfully cloned. *Gn1a* encodes a cytokinin oxidase/dehydrogenase (OsCKX2), an enzyme that degrades the phytohormone cytokinin. Although both *Gn1a*/*OsCKX2* alleles of Habataki and Koshihikari have normal function, the reduced expression of *Gn1a*/*OsCKX2* in Habataki causes cytokinin accumulation in inflorescence meristems and increases the number of reproductive organs, resulting in enhanced grain yield. Most importantly, there is no difference in grain size between NILs carrying different *Gn1* alleles. Thus, *Gn1* is a useful locus for increasing grain productivity (Ashikari et al. 2005).

11.2.14 *IDEAL PLANT ARCHITECTURE 1*

IPA1 (*IDEAL PLANT ARCHITECTURE 1*), also named WFP (*WEALTHY FARMER'S PANICLE*), encodes the transcription factor OsSPL14 which contained the SBP structural domain. IPA1/WFP was located in the nucleus with the transcriptional activity. Analysis of mRNA in situ expression showed that *IPA1*/*WFP* had the highest expression in stem tips during vegetative growth period and branch primodium during reproductive growing period. It was found that either a C-to-T SNP in the coding region of *IPA1* that affects miR156 targeting (named *ipa1-1D* allele) or increased *IPA1* expression in the panicles due to epigenetic regulation in its upstream promoter region (named $IPA1^{WFP}$allele) conferred an ideal plant architecture for rice, including fewer tillers, thick stem, and obvious increase in grain number per spike and 1000-grain weight (Jiao et al. 2010; Miura et al. 2010). Subsequent studies have found that IPA1 could directly bind to the GTAC motif in the promoters of *FINE CULM 1* (orthologous to the maize *TEOSINTE BRANCHED1*, also named *OsTB1*) and *DENSE AND ERECT PANICLE1* (*DEP1*), which are known key regulators of tillering and panicle morphology, respectively (Lu et al. 2013). So *IPA1* is a powerful gene for improving the plant type of current rice cultivars and enhancing the rice yield with great application potential in rice breeding.

11.3 Networking the Genes for Rice Grain and Number

The isolation and functional analysis of many QTL genes for rice grain size and number have provided important clues for the discovery of the networks involved in this key agronomic trait. Currently available evidence suggests that grain size and number are controlled by multiple signaling pathways involving phytohormone and G-protein signaling pathways (Fig.11.1).

11.3.1 Plant Hormones

Plant hormones regulate nearly all aspects of plant growth including seed development. Several phytohormones such as brassinosteroid (BR), auxin, and cytokinin have been suggested to play an important role in seed growth. Emerging evidence also uncovers a regulatory mechanism on seed size control possibly mediated by the interaction of BR and G-protein signaling.

11.3.1.1 Auxin

Auxin regulates almost every aspect of plant growth and development. Among the identified QTL discussed above, *TGW6*, a major QTL that negatively controls rice grain weight and grain filling, encodes an IAA-glucose hydrolase and plays an important role in the regulation of auxin homeostasis during endosperm development (Ishimaru et al. 2013). Activation of rice *BIG GRAIN1* (*BG1*), which is

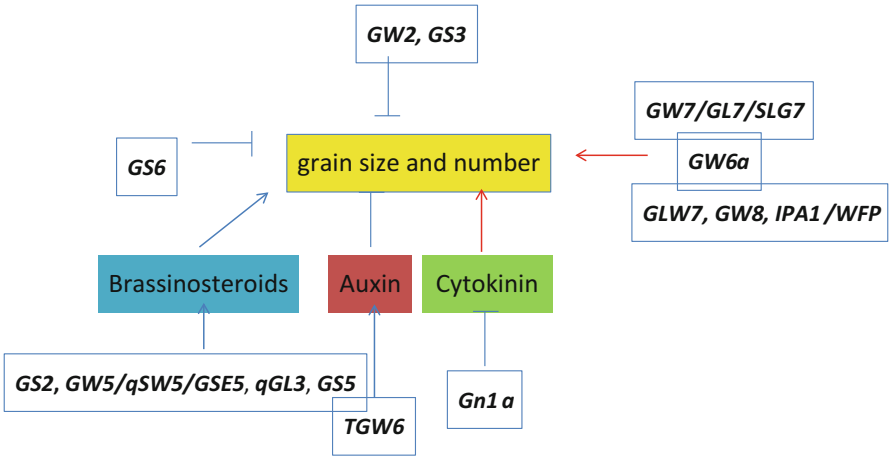

Fig. 11.1 Major regulatory QTL/genes that control grain size and number

involved in auxin transport, results in large grains due to increased cell proliferation and cell expansion in spikelet hulls (Liu et al. 2015). Thus, auxin biosynthesis, transport, and signaling seem to be important for seed size control.

11.3.1.2 Cytokinin

Cytokinins have been reported to influence seed growth and development. The rice *Gn1a* encodes a cytokinin oxidase/dehydrogenase (OsCKX2) that irreversibly catalyzes degradation of cytokinin and is preferentially expressed in the inflorescence meristem and flowers (Ashikari et al. 2005). Mutations in *Gn1a*/*OsCKX2* and *DROUGHT AND SALT TOLERANCE* (*DST*), which encodes a zinc finger transcription factor directly and positively regulating the expression of *Gn1a*/*OsCKX2* (Li et al. 2013), cause the increased accumulation of cytokinin in the inflorescence meristem and, consequently, the increased grain number. In general, the increased number of seeds or grains is closely associated with the reduced size or vice versa, presumably owing to the availability of the fixed carbon in the source and the efficiency of transport to the sink (Huang et al. 2009; Zhang et al. 2012). However, grain size is also positively correlated with the grain number in some cases. The DST^{reg1} mutant allele causes the increased cytokinin level in the inflorescence meristem, resulting in the significant increase of both the grain number and grain weight (Li et al. 2013). An additional example is the rice *larger panicle* (*lp*) mutant, in which the reduced expression of *OsCKX2* correlates with the increase in both grain number and grain weight (Li et al. 2011b), suggesting that cytokinin positively regulates these two traits.

11.3.1.3 Brassinosteroid

Brassinosteroids (BRs) are a class of growth-promoting steroidal hormones that were initially isolated from rapeseed (*Brassica napus*) pollen, which is crucial for normal growth and development, such as plant height, leaf angle, panicle architecture, and seed size (Wu et al. 2016). In rice, four QTL genes for grain size (*GS3*, *GS5*), grain length (*qGL3*/*GL3.1*), and grain width/weight (*GW5*/*qSW5*) might be involved in BR signaling. *GS5* encodes a putative serine carboxypeptidase, and higher expression of *GS5* results in wide and heavy grains as a result of increased cell proliferation and expansion in spikelet hulls (Li et al. 2011a). *GS5* was reported to competitively inhibit the interaction between OsBAK1-7 and OsMSBP1 (Xu et al. 2015), suggesting that *GS5* might influence BR signaling. *qGL3*/*GL3.1* encodes a protein phosphatase with Kelch-like repeat domains (OsPPKL1) and specifically affects grain length (Hu et al. 2012; Qi et al. 2012; Zhang et al. 2012). OsPPKL1 shares similarity with *Arabidopsis* AtBSU1 and AtBSL1 (Zhang et al. 2012). AtBSU1 and its homolog AtBSL (BSU1-like) directly dephosphorylates the

GSK3-like kinase BIN2 (BR-insensitive 2) or antagonizes the BIN2 activity on a downstream component to positively regulate BR signaling (Mora-Garcia et al. 2004; Kim et al. 2009), suggesting that *qGL3* might participate in BR signaling in rice.

GS2, a plant-specific transcription factor *OsGRF4* that is targeted by miR396, predominantly promotes cell expansion but slightly increases cell proliferation in spikelet hulls (Hu et al. 2015; Duan et al. 2015; Che et al. 2015). Expression of *GS2*/*OsGRF4* is regulated by OsmiR396 in rice. The rare allele $GS2^{AA}$ from large-grain varieties disrupts OsmiR396-directed regulation of *GS2*, resulting in heavy grains and increased grain yield in rice (Duan et al. 2015; Che et al. 2015). OsGSK2 (GSK3/SHAGGY-like kinase 2), a negative regulator of BR signaling, interacts with GS2 and inhibits its transcriptional activation activity, suggesting that BR might be involved in GS2-mediated grain size control (Che et al. 2015). Using a carboxyl-terminal fragment of GW5 as the bait to screen a yeast two-hybrid library, GSK2 was identified as a viable interacting partner of GW5, which was confirmed by BiFC and pull-down assays (Liu et al. 2017). Consistently, in the lamina joint inclination assays, transgenic rice plants overexpressing *GW5* in the genetic background of *GSK2* RNAi transgene greatly enhanced the phenotypes, which exhibited hypersensitivity to exogenously applied brassinolides (Liu et al. 2017). In addition, a series of biochemical analyses indicated that GW5 could repress the kinase activity of GSK2 toward OsBZR1 (*Oryza sativa* BRASSINAZOLE RESISTANT1) and DLT (DWARF AND LOW-TILLERING), resulting in accumulation of their unphosphorylated forms and altered BR signaling (Liu et al. 2017). Therefore, GW5 might be a positive regulator of the BR signaling pathway in regulating grain width and weight in rice.

11.3.2 G-Protein Signaling

G-protein signaling is involved in diverse growth and developmental processes in plants and animals. G-protein-coupled pathways transmit a signal, via a membrane receptor and heterotrimeric G-protein complex consisting of Gα-, Gβ-, and Gγ-subunits, to downstream effectors. The loss-of-function mutations in the putative γ-subunit gene *GS3* increase grain size (Fan et al. 2006; Mao et al. 2010). Conversely, overexpression of the OSR domain of *GS3* or a gain-of-function mutation in another γ-subunit gene *DEP1* (the *dep1* allele), which maintains the intactness of the inhibitory OSR domain, results in the formation of smaller grains than the wild type (Huang et al. 2009; Mao et al. 2010). It appears that the rice γ-subunits GS3 and DEP1 function distinctively from their *Arabidopsis* partner AGG3 (Li et al. 2012) and the α- and β-subunits of rice G-protein (RGA1 and RGB1) (Ashikari et al. 1999; Fujisawa et al. 1999; Utsunomiya et al. 2011). It is plausible that GS3 and DEP1 in rice and AGG3 in *Arabidopsis* might possess different cofactors or

effectors, resulting in opposite effects on seed size. Further studies are required to identify the effectors or downstream components of G-protein subunits in seed size control.

11.4 Conclusions and Prospects

Although great progress has been made in understanding of the molecular mechanisms controlling rice grain size and number, our current knowledge on the two critical agronomic traits is rather fragmented, as the regulatory pathways involved in grain size and number are full of gaps. The interactions between different pathways are largely elusive. In rice, several important factors controlling grain size and number have been identified, but their genetic relationships are largely unknown. There are also some inconsistent results. The main reason is that the mutant alleles for grain size and number are in different rice varieties. Rice scientists usually use near-isogenic lines, which might contain a number of other mutations, to conduct physiological and genetic analyses. The use of newly developed genome-editing technologies to generate mutants for known seed size genes in the same genetic background will facilitate establishing of genetic networks controlling seed size and number in rice or other crops. It is noteworthy that the same mutant allele might cause different phenotypes in different genetic backgrounds in rice (Huang et al. 2009; Zhou et al. 2009). Understanding why the same mutation works differently in different varieties will help breeders to rationally utilize the mutant alleles to improve seed yield in a specific genetic background.

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Chapter 12
Domestication Loci Controlling Panicle Shape, Seed Shattering, and Seed Awning

Takashige Ishii and Ryo Ishikawa

Abstract Cultivated rice (*Oryza sativa* L.) was domesticated from the Asian wild species, *Oryza rufipogon* Griff. Among morphological differences between them, one of the striking traits specific to cultivated rice is loss of seed shattering. In the early stage of rice domestication, the related traits of this character have been desirable for the ancient seed gatherers because it enhances the efficiency of seed collection. In this chapter, we propose that three morphological traits, closed panicle shape, non-seed shattering, and seed awning, played important roles in controlling the degree of seed dispersal. First, we reviewed domestication loci controlling the three traits. We then evaluated allele effects at these loci using reciprocal backcross populations between *O. sativa* Nipponbare and our standard wild accession of *O. rufipogon* W630. In the genetic background of cultivated rice, all the wild functional alleles were responsible for these domestication traits. On the other hand, cultivated non-functional alleles were not always associated with the drastic morphological changes in the genetic background of wild rice. Since ancient humans have selected cultivated-type mutants in natural wild populations, possible domestication process for the emergence of cultivated rice is discussed based on the effects of cultivated non-functional alleles.

Keywords Domestication · *Oryza rufipogon* · Panicle spreading · Seed shattering · Seed awning

12.1 Introduction

Cultivated rice (*Oryza sativa* L.) was domesticated from the Asian wild species, *Oryza rufipogon* Griff (Fig. 12.1) (Oka 1988). Several distinct morphological differences are observed between them, of which one of the striking traits specific

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Fig. 12.1 Asian wild rice, *Oryza rufipogon*. This wild species is commonly found in swampy and deepwater areas in tropical Asian countries

to cultivated rice is loss of seed shattering. The related traits of this character have been desirable for the ancient seed gatherers because they enhance the efficiency of seed collection. When planting what they had harvested started, artificial selection was imposed on the shattering-related traits of rice plants. Therefore, it is considered that reduction of the seed-shattering degree is required in the early phase of domestication (Flannery 1973; Harlan et al. 1973; Fuller 2007).

Compared with cultivated rice, wild rice produces spikelets with long awns (bristle-like organs in the tip of the lemma) on spreading panicles (Fig. 12.2), and the seeds mature from top to bottom of the panicle. About 2 weeks after flowering, an abscission layer is completely formed between the pedicel and spikelet, and the seeds shed by themselves. Open panicle shape and long awns also enhance dispersal of mature seeds. Recently, we found a simple morphological change in panicle shape had a large impact on the seed-shedding behavior of wild rice (Fig. 12.3) (Ishii et al. 2013). In the maturing stage, wild rice plants with a cultivated-type closed panicle had significantly reduced seed shedding. There was a tendency to retain the upper mature seeds on the panicles through support from long awns in the lower immature seeds. In addition, the long awns in closed panicles inhibited the free exposure of anthers and stigmas on the flowering spikelets, resulting in a significant reduction in the outcrossing rate. These observations suggest that closed panicle shape and long awns played important roles in seed-shedding and pollinating behaviors during the early stage of rice domestication. We do not know the exact historical sequence of rice domestication, but three traits, i.e., closed panicle shape, non-seed shattering, and seed awning are closely associated with the emergence of cultivated rice. In this chapter, the genetic mechanisms of these three domestication traits are reviewed and discussed.

Fig. 12.2 Panicles of cultivated rice, *O. sativa*, and Asian wild rice, *O. rufipogon*. Wild rice has awned spikelets and spreading panicles

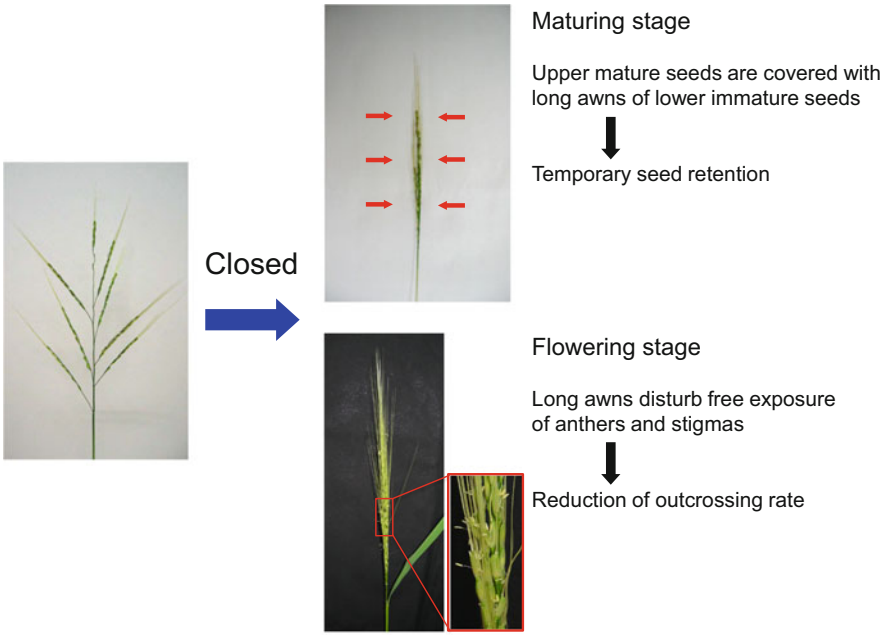

Fig. 12.3 A simple morphological change of panicle shape has a big impact on seed-shattering and pollinating behaviors of wild rice. Closed panicles in wild rice cause temporary seed retention in the maturing stage and reduction of outcrossing rate in the flowering stage

12.2 A Locus Responsible for Panicle Spreading

Panicles of cultivated rice are closed, whereas most Asian wild rice have spreading panicles. The morphological differences in panicles between cultivated and wild rice are mainly explained by a single locus, *SPR3* (*spreading panicle-3*), where wild rice has the dominant allele for spreading panicles (Eiguchi and Sano 1990).

To examine morphological and genetic mechanisms of panicle structure, we previously investigated two plant materials, a cultivar of *O. sativa* Nipponbare (with closed panicles) and a wild accession of *O. rufipogon* W630 (with spreading panicles) (Ishii et al. 2013). The panicle shape of the cultivated and wild accessions is formed at the basal structure in the primary branches. At the cellular level, *O. rufipogon* W630 has tissue resembling a bump between the main and primary branches (Fig. 12.4). To confirm the chromosomal location of the panicle-spreading locus, we performed quantitative trait locus (QTL) analysis using 161 BC_2F_8 plants between *O. sativa* Nipponbare (recurrent parent) and *O. rufipogon* W630 (donor parent). One strong QTL corresponding to *SPR3* was detected on the long arm of chromosome 4, which explained 80.1% of the phenotypic variance. Further fine mapping narrowed down the location of *SPR3* to a 9.3-kb region. Although no coding sequences were predicted in this region, complementation tests showed that the region regulates the rice *LIGULELESS1* gene (*OsLG1*), Locus ID Os04g0656500 in the Rice Annotation Project (RAP) Database (Fig. 12.5, Table 12.1). *OsLG1* is located 10 kb away from the *SPR3* locus and encodes a *SQUAMOSA* promoter binding protein (SBP) domain (Lee et al. 2007).

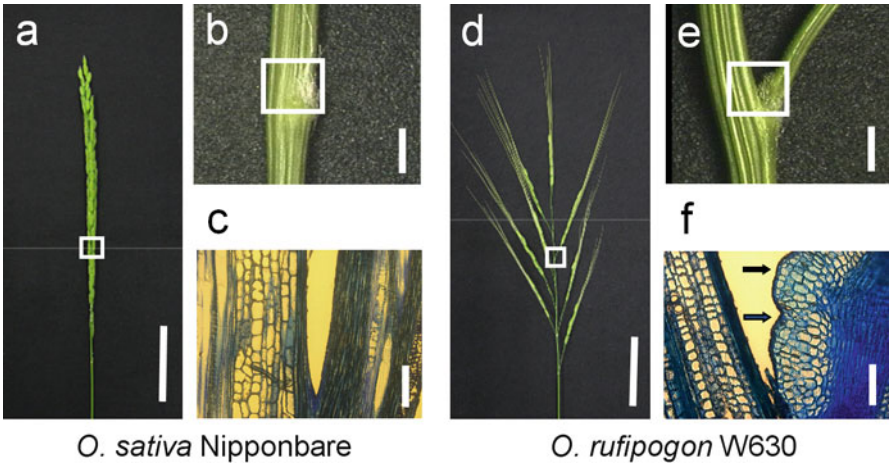

Fig. 12.4 Panicles of *O. sativa* Nipponbare (**a–c**) and *O. rufipogon* W630 (**d–f**) (Modified from Ishii et al. 2013). (**a, d**) Panicles in the heading stage. Scale bar, 5 cm. (**b, e**) Basal structure of the primary branches indicated by white boxes in (**a**, **d**). Scale bar, 1 mm. (**c, f**) Longitudinal sections of the basal parts of primary branches indicated by white boxes in (**b**, **e**). Arrows show the bump structure tissue. Scale bar, 100 μm

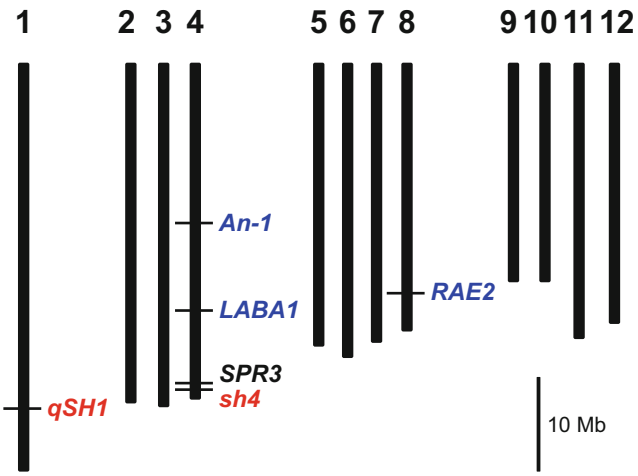

Fig. 12.5 Chromosomal positions of loci for panicle spreading (*SPR3* in black), seed shattering (*sh4* and *qSH1* in red), and seed awning (*An-1*, *LABA1*, and *RAE2* in blue). Their physical positions are after the Rice Annotation Project Database (IRGSP-1.0)

Furthermore, it controls laminar joint and ligule development. The physical position is about 33.49 Mb on chromosome 4 according to the RAP Database. This gene was also named *OsSPL8*, one of the SBP-box genes in rice (Yang et al. 2008). In the basal parts of primary branches, higher levels of *OsLG1* expression were observed in *O. rufipogon* W630 than in *O. sativa* Nipponbare (Ishii et al. 2013). In addition, overexpression of *OsLG1* has been reported to induce the spreading panicle phenotype in transgenic lines (Zhu et al. 2013). Since the panicle phenotypes corresponded to the expression levels of *OsLG1*, we concluded that the *SPR3* region contained the regulatory sequences of *OsLG1* expression in the basal parts of the primary branches.

12.3 Allele Effects at Panicle-Spreading Locus

Among the 161 BC_2F_8 plants between *O. sativa* Nipponbare and *O. rufipogon* W630 used in the above QTL analysis, all the plants having wild W630 alleles at *SPR3* had spreading panicles. This demonstrates that wild alleles are responsible for panicle spreading in the genetic background of cultivated rice. We developed separately an introgression line that contained a chromosomal segment from *O. sativa* Nipponbare at the *SPR3* region in the genetic background of *O. rufipogon* W630. This line had closed panicles with no bump structure tissue

Table 12.1 Domestication loci controlling panicle spreading, seed shattering, and seed awning

Trait	Locus	Chr.	Physical location[a] (Mb)	Mutated region	Responsible gene locus ID[a]	Description[a]	References
Panicle spreading	*SPR3*	4	33.49	Regulatory region of *OsLG1*	Os04g0656500	Similar to Squamosa-promoter binding-like protein 3	Ishii et al. (2013)
Seed shattering	*sh4*	4	34.23	Coding region	Os04g0670900	Homeodomain-like containing protein	Li et al. (2006)
	qSH1	1	36.45	Regulatory region of *OsRPL*	Os01g0848400	BEL1-type homeobox family	Konishi et al. (2006)
Seed awning	*An-1*	4	16.73	Coding region	Os04g0350700	Basic helix-loop-helix protein	Luo et al. (2013)
	LABA1	4	25.96	Coding region	Os04g0518800	Cytokinin synthesis enzyme	Hua et al. (2015)
	RAE2	8	23.99	Coding region	Os08g0485500	Epidermal patterning factor-like 1 protein	Bessho-Uehara et al. (2016)

[a]After the Rice Annotation Project Database (IRGSP-1.0)

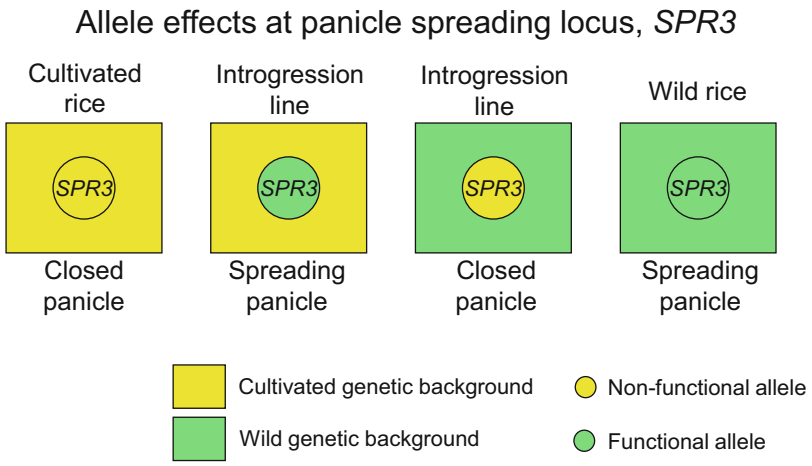

Fig. 12.6 Schematic representation of allele effects at panicle-spreading locus, *SPR3*, in the cultivated and wild genetic backgrounds

at the base of the primary branches, indicating that closed panicle shape is simply generated by a loss-of-function mutation at *SPR3*. The effects of the non-functional cultivated and functional wild alleles at *SPR3* are shown in Fig. 12.6. We therefore infer that ancient humans may have selected closed panicle plants with the non-functional allele at *SPR3* in natural wild populations.

12.4 Closed Panicle Influences Seed-Shedding and Outcrossing Behaviors in Wild Rice

To evaluate the effects of the closed panicle on seed-shedding and outcrossing behaviors in wild rice, the introgression line containing the Nipponbare allele in the genetic background of *O. rufipogon* W630 was produced (Ishii et al. 2013). For seed-shedding behavior, a seed-gathering experiment using the introgression line and *O. rufipogon* W630 was carried out in the field. Since both plants display a seed-shattering habit, their seeds were collected directly from the panicles by hand in the maturing stage, and seed-gathering rates were calculated. Significantly, more seeds (mean gathering rate = 30.6%) were collected from the introgression line than the wild parental accession of *O. rufipogon* W630 (19.8%), indicating that seeds could be gathered more efficiently from the closed panicles (Ishii et al. 2013). Furthermore, to confirm that the plants with closed panicles can retain mature seeds longer than those with open panicles, days from flowering to seed shedding were evaluated with the introgression line and the wild parent. A significant difference was observed for the average days between the introgression line (14.8 days) and *O. rufipogon* W630 (13.8 days). This indicates that plants with closed panicles

could retain mature seeds for about 1 day longer than those with open panicles and explains why mature seeds were efficiently collected from the plants with closed panicles.

The outcrossing rates between the introgression line and two wild lines of *O. rufipogon* W630 were also examined (Ishii et al. 2013). All plant material displayed a similar wild morphology, except for the closed panicles observed in the introgression line. Outcrossing rates of the two wild lines were 10.24% and 11.71%, whereas that for the introgression line was 2.82%. A significant reduction in outcrossing rate was caused by a single non-functional allele at *SPR3* which changed panicle structure from open to closed. This morphological change may have a big impact on pollination behavior during rice domestication.

12.5 Loci Responsible for Seed Shattering

Wild rice, *O. rufipogon*, has strong seed-shattering behavior that guarantees propagation success through seed dispersal under natural condition. A wide variation in seed-shattering degree is also observed among cultivated rice, *O. sativa*, suggesting that seed shattering is a quantitatively regulated trait. Two loci, *sh4* and *qSH1*, were reported to have a strong influence on the shattering habit (Konishi et al. 2006; Li et al. 2006). The *sh4* is located on the long arm of chromosome 4 with a physical position of about 34.23 Mb (just 0.74 Mb distal to *SPR3* locus) (Fig. 12.5, Table 12.1). It was detected as a major QTL for seed shattering between *O. rufipogon* and *O. sativa* Indica and explains 69% of the total phenotypic variance in their segregating populations. A gene responsible for *sh4* encodes a Myb-type transcription factor (Locus ID Os04g0670900). An allele with a loss-of-function mutation was found to inhibit the establishment of the abscission layer at flower development stage (Li et al. 2006). Since the loss-of-function mutation is commonly observed in cultivated rice, it is widely accepted that the mutation played an important role in rice domestication (Lin et al. 2007; Onishi et al. 2007; Zhang et al. 2009).

The other major QTL is *qSH1*, which was identified on chromosome 1 (ca. 36.45 Mb) using a segregating population between *O. sativa* Indica Kasalath and Japonica Nipponbare (Konishi et al. 2006) (Fig. 12.4, Table 12.1). A causative mutation at *qSH1* was shown to be an SNP located 12 kb upstream of the rice homolog of the *Arabidopsis REPLUMLESS* (*RPL*) gene for dehiscence zone formation in the *Arabidopsis* silique. It belongs to the BEL1-type homeobox family (Locus ID Os01g0848400). The Nipponbare allele causes the reduction of rice *RPL* gene expression, resulting in the complete absence of abscission layer formation. This causative mutation is attributed to selection for the strong non-shattering behavior of Japonica cultivars.

12.6 Allele Effects at Seed-Shattering Loci

In both studies on seed-shattering loci, the functional alleles were found to be responsible for the formation and development of an abscission layer between the pedicel and spikelet in the genetic background of the cultivars (Konishi et al. 2006; Li et al. 2006).

We evaluated allele effects at these two loci using reciprocal backcross populations between *O. sativa* Japonica Nipponbare (with non-functional alleles) and our standard wild accession of *O. rufipogon* W630 (with functional alleles) (Ishikawa et al. 2010). We define the degree of seed shattering into three categories: non-seed shattering, weak seed shattering (mature seeds can be detached by hand gripping), and seed shattering (no mature seeds remained on the panicles). In the genetic background of Nipponbare, both wild alleles at *sh4* and *qSH1* generated plants with weak seed-shattering behavior (Fig. 12.7). These plants retain mature seeds on the panicles unless external force, such as hand gripping, is added. Strong seed shattering was observed in plants having wild alleles at both *qSH1* and *sh4*. In the genetic background of wild rice, effects of the Nipponbare alleles were also examined (Fig. 12.7). It was serendipitous that the backcross plants having the Nipponbare alleles at either shattering locus (*qSH1* or *sh4*) shed all seeds. This

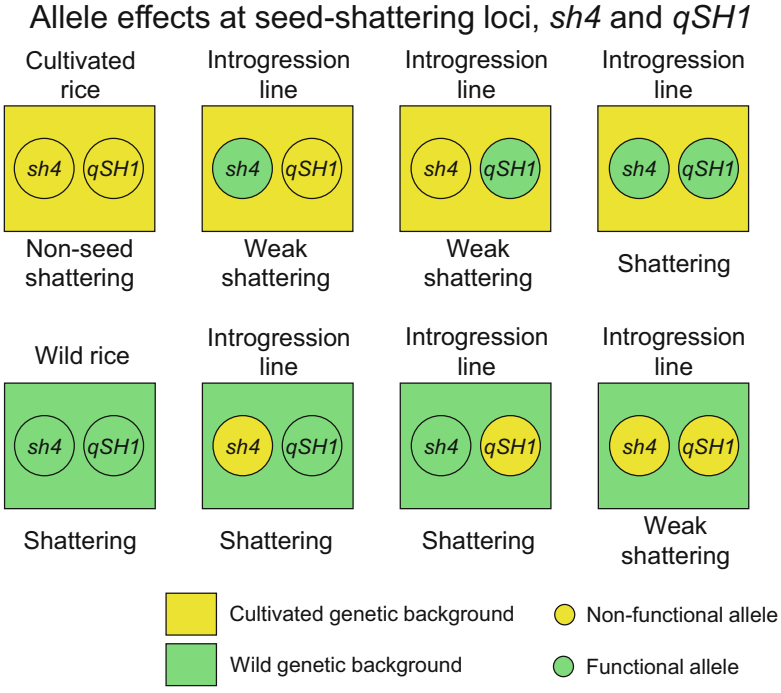

Fig. 12.7 Schematic representation of allele effects at seed-shattering loci, *sh4* and *qSH1*, in the cultivated and wild genetic backgrounds

suggests that a non-functional allele at *qSH1* or *sh4* does not contribute to non-seed shattering in the genetic background of wild rice. The plants having the Nipponbare alleles at both *qSH1* and *sh4* retained mature seeds on the panicles; however, the seeds easily detached by hand tapping. This also confirms that non-shattering behavior is not obtained by a single mutation at these loci in the genetic background of wild rice. Probably, some other loci are still associated with non-shattering behavior of cultivated rice.

12.7 Loci Responsible for Seed Awning

Of the wild propagation-related traits, seed awning directly enhances seed dispersal together with seed-shattering behavior. The major seed-shattering loci have already been identified (Konishi et al. 2006; Li et al. 2006), whereas those for seed awning have been well-characterized only in the past few years. The first reported locus was *Awn-1* (*An-1*) on chromosome 4 with the physical position of about 16.73 Mb in the RAP Database (Luo et al. 2013) (Fig. 12.5, Table 12.1). It was identified as a major QTL explaining 52% of the total phenotypic variance in the segregating population between *O. rufipogon* (long awn) and *O. sativa* (awnless). The responsible gene encodes a basic helix-loop-helix transcription factor (Locus ID Os04g0350700). In general, two types of mutations at the locus were observed among cultivars. Most Japonica cultivars have a transposon insertion in the promoter region, whereas most Indica cultivars possess a 1-bp deletion in the second exon which leads to a premature stop codon.

Another identified locus is *long and barbed awn1* (*LABA1*) being located on chromosome 4 in the region of about 25.96 Mb, which encodes a cytokinin-activating enzyme (Locus ID Os04g0518800) (Hua et al. 2015) (Fig. 12.5, Table 12.1). This locus is associated with the transition from long barbed awns in wild rice to short barbless in Indica rice. A frame-shift 1-bp deletion in the coding region causes a premature stop codon and reduces the cytokinin concentration in awn primordia. This loss-of-function mutation is mainly observed in barbless Indica and tropical Japonica cultivars.

A *regulator of awn elongation 2* (*RAE2*) locus regulates awn elongation in association with the *epidermal patterning factor-like* protein (Bessho-Uehara et al. 2016) (Fig. 12.5, Table 12.1). The locus ID is Os08g0485500 being located in the region of about 23.99 Mb on chromosome 8. In the second exon, a highly variable GC-rich region was found to harbor multiple independent mutations in cultivated rice plants. The RAE2 proteins are mainly classified into three types according to the number of cysteine residues: 4C, 6C, and 7C types. Of these, 6C is a functional type in wild rice, and putatively dysfunctional 4C and 7C types are mainly observed in Japonica and Indica cultivars, respectively.

Two loci of *Awn-2* (*An-2*) and *grain number, grain length, and awn development 1* (*GAD1*) were independently reported to be responsible for awn development (Gu et al. 2015; Jin et al. 2016). The *An-2* and *GAD1* loci are identical to *LABA1* and *RAE2*, respectively.

12.8 Allele Effects at Seed-Awning Loci

We first performed QTL analysis for seed awning using 161 BC_2F_8 plants between *O. sativa* Nipponbare (recurrent parent without awns) and our standard wild accession of *O. rufipogon* W630 (donor parent with long awns). Two strong QTLs were detected on chromosomes 4 and 8 (Ikemoto et al. 2017). Subsequent causal mutation survey and fine mapping confirmed that they are identical to *An-1* and *RAE2* loci, respectively. Regarding *LABA1*, no QTL was detected in the chromosomal region near the locus. This was expected because Nipponbare has a functional allele at *LABA1* just like other wild rice accessions (Hua et al. 2015). We then developed two backcross populations with reciprocal genetic backgrounds of Nipponbare and W630 to examine the allele effects and interactions at major seed-awning loci, *An-1* and *RAE2*.

Awn length in wild rice varied among seeds even in the same plant. We noticed that the seed (or spikelet) position in a panicle affected awn length. Since wild rice exhibits seed-shattering behavior, awn lengths of *O. rufipogon* W630 were compared with the five upper spikelets on the top primary branch just after heading (Ikemoto et al. 2017). The results show that the second spikelet had a significantly shorter awn than the others, and the first and the third followed with similar values (Fig. 12.8). The fifth spikelet always gave a longer awn than the fourth on the same primary branches. Therefore, in this study, the gene effects and interactions were evaluated based on the awn length of the fifth spikelet which generates the longest awn among the five.

A BC_3F_2 population segregated at *An-1* and *RAE2* was produced from the BC_2F_8 plants in the genetic background of Nipponbare to investigate gene interaction. In the BC_3F_2 population, we selected introgression plants with three combinations of genotypes on wild alleles, namely, wild homozygotes at single or both awning loci. Their awn lengths were measured based on spikelet position and compared with the parental accessions (Ikemoto et al. 2017). About half-length awns were observed in the plants having wild alleles at *An-1* (40% in length compared to the wild) and *RAE2* (51%). This suggests their wild alleles had awning effects in the genetic background of Nipponbare (Fig. 12.9). Additive effects were detected in the plants having wild alleles at both loci, and these produced awns whose length was about 78% of the W630 lengths.

In the genetic background of wild rice, allele effects of Nipponbare were examined using the other BC_3F_2 backcross population between *O. sativa* Nipponbare (donor parent) and *O. rufipogon* W630 (recurrent parent). Surprisingly, the plants having Nipponbare non-functional alleles at *RAE2* showed almost the

Fig. 12.8 Panicle and spikelet morphology of *O. rufipogon* W630. (**a**) A panicle of *O. rufipogon* W630. A white box indicates the top primary branch in the panicle. (**b**) Spikelets from the top primary branch of *O. rufipogon* W630. They were arranged according to their positions from the top (first to fifth spikelets). Scale bar, 5 cm

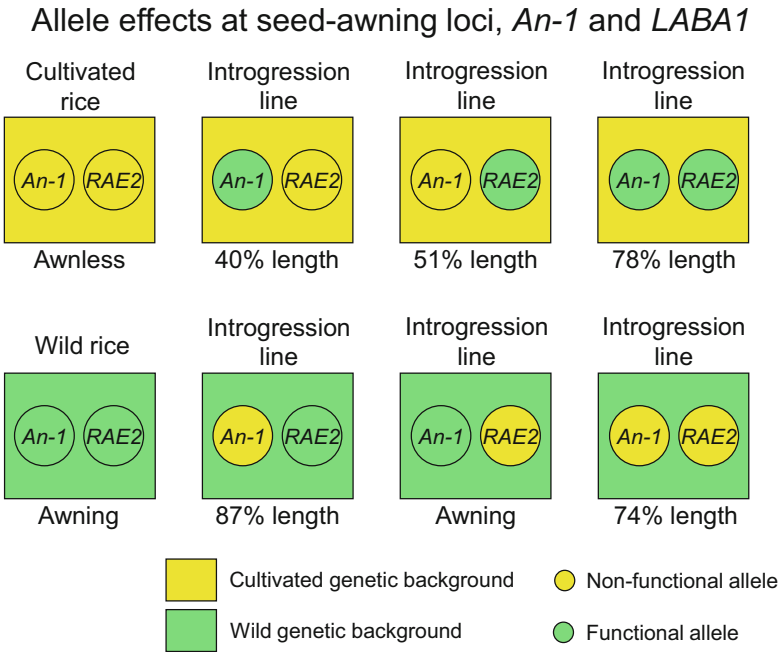

Fig. 12.9 Schematic representation of allele effects at seed-awning loci, *An-1* and *LABA1*, in the cultivated and wild genetic backgrounds

same awn lengths as those of W630 (Fig. 12.9). This suggests that the substitution of the non-functional alleles at *RAE2* did not contribute to length reduction at all. But it was observed that the plants with Nipponbare alleles at *An-1* had significantly shorter awns where the length reduction rate was approximately 13%. Even the plants having the Nipponbare non-functional alleles at both loci produced long awns (corresponding to about 74% of the W630 lengths). These results show that more loci other than *An-1* and *RAE2* are expected to contribute to awnlessness in the genetic background of wild rice.

12.9 Possible Domestication Process for the Emergence of Cultivated Rice

Loss of seed shattering is a key trait for the emergence of cultivated rice, because it enhanced the efficiency of seed collection by ancient seed gatherers. However, it is difficult to find non-shattering plants among wild rice populations. Non-shattering plants are easily eliminated under natural conditions due to their lower propagation ability. In addition, the appearance of non-shattering behavior probably requires independent mutations at multiple seed-shattering loci in the genetic background of wild rice. On the other hand, the temporary inhibition of seed shedding can be obtained through a simple morphological change in panicle shape. Wild rice has spikelets with long awns and spreading panicles, but a single mutation at *SPR3* produces plants with closed panicles. They tend to retain mature seeds a little longer on the panicles with the support from the long awns of immature seeds. After maturity, all the seeds are shed without changing their wild propagation ability. Therefore, a closed panicle may be a first key trait in the development of cultivated rice (Fig. 12.10).

Furthermore, the long awns in closed panicles disturb the free exposure of anthers and stigmas on the flowering spikelets, resulting in a significant reduction of the outcrossing. This morphological change of the panicles promotes self-pollinating behavior and fixation of genes and may assist in the accumulation of homozygous alleles of recessive mutations on seed shattering. Once non-seed-shattering plants are generated in the early stage of rice domestication, seed awning may have been an undesirable trait during harvesting and rice-processing activities. Although seed awning in the genetic background of wild rice is under complex control, an awnless phenotype may be gradually generated by the accumulation of recessive alleles at seed-awning loci.

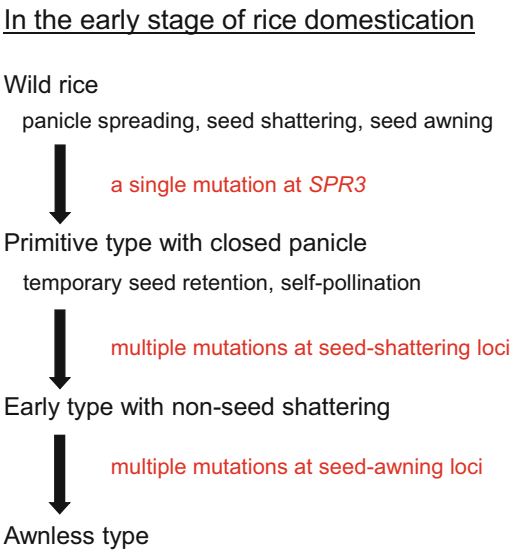

Fig. 12.10 Possible domestication process for the emergence of cultivated rice

12.10 Perspective

Wild rice keeps many functional alleles at propagation-related loci to survive under natural condition, whereas cultivated rice has accumulated loss-of-function mutations at these loci. In rice domestication, the ancient humans selected desirable mutant plants in the wild natural populations. Therefore, to investigate the transition of the domestication traits, the effects of non-functional cultivated alleles should be examined in the wild genetic background. The non-functional alleles are not always associated with the drastic morphological changes, because many other wild genes are generally involved in the trait expression. Studies on gene interaction at multiple domestication loci in the wild genetic background will give good clues to elucidate the process of rice domestication.

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Chapter 13
Transport System of Mineral Elements in Rice

Namiki Mitani-Ueno, Naoki Yamaji, and Jian Feng Ma

Abstract Plant requires 14 mineral elements for their growth and development. These elements in the soil are taken up by the roots, translocated from the roots to the shoots, and distributed to different organs depending on their demands (Marschner P, Mineral nutrition of higher plants, 3rd edn. Academic, London, 2012). In addition to these essential elements, toxic elements such as Cd and As are also transported from the soils to aboveground parts. All these processes require various transporters (membrane proteins). During the last decades, a number of transporters for uptake, translocation, and distribution of mineral elements have been identified, especially in model plants such as *Arabidopsis* and rice; however, most transporters remain to be identified. In this chapter, transporters identified so far in rice are described, and the regulation mechanisms of transporters in response to environmental changes are also discussed.

Keywords Transporter · Mineral elements · Uptake · Distribution

13.1 Brief Introduction of the Root and Node Structure in Rice for Mineral Transport

Rice grown in paddy field shows a distinct transport system for uptake and distribution due to the structural features of its roots and nodes. Rice roots are characterized by two Casparian strips at both the exodermis and endodermis (Enstone et al. 2002). Furthermore, mature roots have a highly developed aerenchyma in which almost all of the cortex cells between the exodermis and endodermis are destroyed. Therefore, movement from the soil solution to the stele requires both influx and efflux transporters in both the exodermis and endodermis of the roots (Sasaki et al. 2016, Fig. 13.1).

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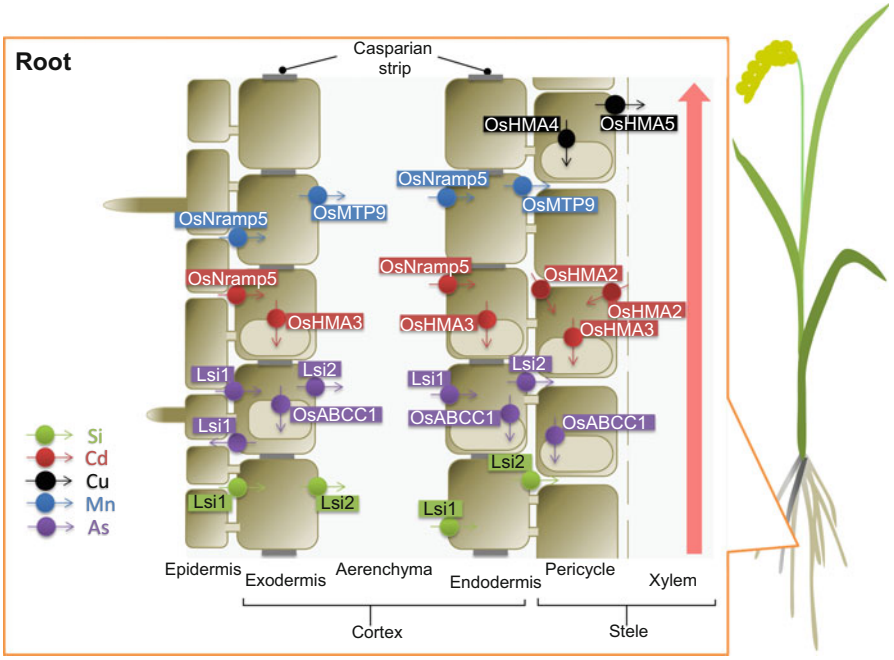

Fig. 13.1 Schematic diagram of transporters identified so far for uptake and sequestration of essential and toxic elements in rice roots. Pink arrow indicates xylem flow; green Si channel Lsi1 and efflux transporter Lsi2; red Cd influx transporter OsNramp5 and OsHMA2 and tonoplast-localized transporter OsHMA3 for Cd sequestration; black Cu efflux transporter OsHMA5 and tonoplast-localized transporter OsHMA4 for Cu sequestration; blue Mn influx transporter OsNramp5 and Mn efflux transporter OsMTP9; purple As channel Lsi1, As efflux transporter Lsi2, and tonoplast-localized transporter OsABCC1 for As sequestration

In terms of distribution of mineral elements, rice nodes have been demonstrated to play an important role in the distribution. Rice nodes have highly developed vascular systems, mainly consisting of enlarged vascular bundles (EVBs) and diffuse vascular bundles (DVBs). EVBs come from the two lower nodes and are connected to the leaf attached to the node, while DVBs start at the node and are connected to the upper two nodes or panicle. Therefore, an intervascular transfer of mineral elements from EVBs to DVBs is required for their preferential distribution to developing tissues and reproductive organs. Various transporters for different mineral elements are also required for this intervascular transfer (Fig. 13.2).

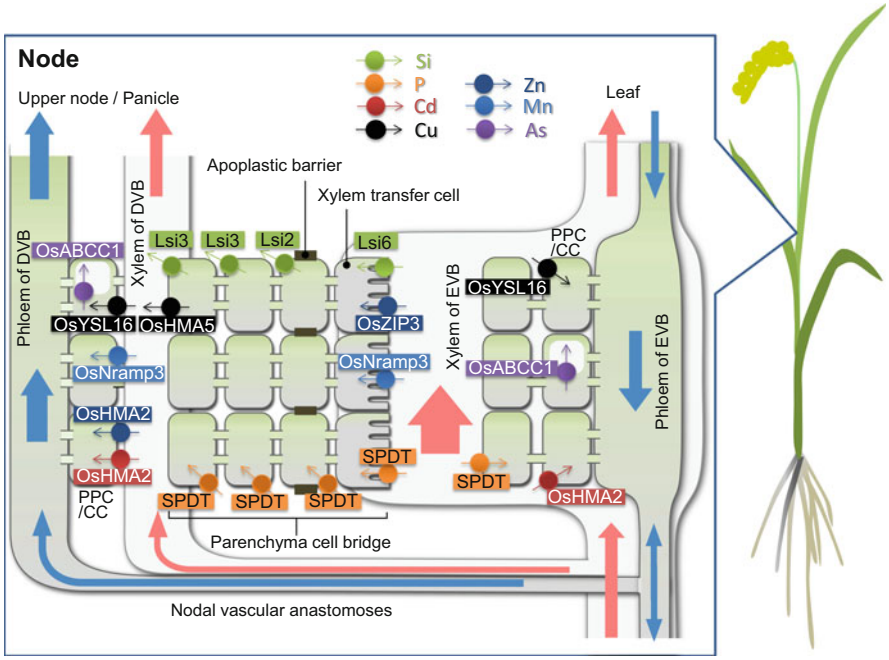

Fig. 13.2 Schematic diagram of transporters identified so far for distribution of essential and toxic elements in rice nodes. Pink and light blue arrows indicate xylem and phloem flow, respectively; green Si channel Lsi6 and Si efflux transporters Lsi2 and Lsi3; orange P influx transporter SPDT; red Cd transporter OsHMA2; black Cu efflux transporter OsHMA5 and Cu-nicotianamine transporter YSL16; dark blue Zn transporters OsZIP3 and OsHMA2; blue Mn influx transporter OsNramp3; purple tonoplast-localized transporter OsABCC1 for As sequestration

13.2 Transporters Involved in Uptake and Distribution of Essential Elements

13.2.1 Transporters for Macronutrients

Plants need six macronutrients (N, P, K, Mg, S, Ca) for their healthy growth. Nitrogen (N) is one of the most important nutrients for rice productivity and can be available in several different forms, including ammonium ions (NH_4^+) and nitrate ions (NO_3^-) and organic molecules, such as amino acids. Rice is usually cultivated under flooded condition, where NH_4^+ is the dominant inorganic N form. For the uptake of NH_4^+, rice has two uptake systems: a high-affinity transport system (HATS) and a low-affinity transport system (LATS) (Wang et al. 1993). Two types of ammonium transporters (AMTs) have been implicated in the ammonium uptake. The first ammonium transporter (AMT1) involved in HATS was identified in *Arabidopsis* (Ninnemann et al. 1994). In rice genome, there are 12 potential AMT members, but only few of them have been characterized.

OsAMT1;2 may play a role in NH_4^+ uptake and xylem loading (Sonoda et al. 2003). *OsAMT1;2* expression is induced by ammonium supply, and its mRNA is detected in the exodermis, sclerenchyma, endodermis, and pericycle cells of primary root tips by in situ hybridization (Sonoda et al. 2003). Two other members of AMT1 such as OsAMT1;1 and OsAMT1;3 were also reported as transporters of NH_4^+ uptake (Hoque et al. 2006; Ferreira et al. 2015); however their exact roles remain to be investigated.

Rice is also able to take up NO_3^-, which is oxidized from ammonium in the rhizosphere of rice through oxygen release from the root aerenchyma (Kirk and Kronzucker 2005). The *indica* cultivars usually have greater nitrate uptake ability than *japonica* cultivars, probably because *indica* cultivars are more frequently cultivated in upland soil where nitrate is the dominant N form. Plant nitrate transporters (NPF) belong to the nitrate transporter 1/peptide transporter (NRT1/PTR) family. There are at least 93 NPF members in the rice genome (Li et al. 2015), but only a few have been characterized. Recently, one gene (NRT1.1B/NPF6.5), responsible for the genotypic difference in nitrate uptake between *japonica* and *indica* cultivars, was identified (Hu et al. 2015; Chen and Ma 2015). It encodes a plasma membrane-localized transporter for nitrate and is mainly expressed in the epidermis, root hairs, and vascular tissues. Furthermore, its expression is induced by nitrate. In addition to NRT1.1B/NPF6.5, a plasma membrane-localized OsNPF2.2 has been implicated in the root-to-shoot translocation of nitrate (Li et al. 2015). It is mainly expressed in the parenchyma cells around the xylem. Disruption of OsNPF2.2 resulted in increased nitrate concentration in the shoot xylem exudate (Li et al. 2015). OsNPF2.2 might be involved in the xylem unloading of NO_3^- and effects on root-shoot NO_3^- translocation. OsNRT2.3a expressed in parenchyma cells around the root xylem is also responsible for long-distance transport of NO_3^- from root to shoot (Tang et al. 2012). OsNRT2.3b expressed in the leaf phloem plays a key role in NO_3^- remobilization and phloem pH balance (Fan et al. 2016).

Phosphorus (P) is also an important nutrient for plant growth and often becomes a limiting factor for crop productivity due to its low availability and strong fixation to soil particles. There are two forms of inorganic P (Pi), $H_2PO_4^-$ and HPO_4^{2-}, depending on the soil pH. The uptake of Pi is mediated by plasma membrane-localized Pi transporters belonging to the phosphate transporter (PHT1/PT) family. Thirteen PHT1 genes have been identified in rice (Raghothama 1999; Goff et al. 2002), and some of them have been implicated in root Pi uptake. OsPht1;4 (PT4) and OsPht1;8 (PT8) are expressed in the roots and show a transport activity of Pi in yeast or oocytes (Jia et al. 2011; Zhang et al. 2015). Phenotypic analysis showed that root and shoot Pi concentration was significantly lower in the knockout/knockdown lines of OsPht1;4 or OsPht1;8 than in that of the wild-type rice (Jia et al. 2011, Zhang et al. 2015), indicating their role in Pi uptake. Two other members, OsPht1;2 (OsPT2) and OsPht1;6 (OsPT6), are expressed in both the roots and shoots (Ai et al. 2009). OsPT2 is localized exclusively to the stele of primary and lateral roots, whereas OsPT6 is expressed in both epidermal and cortical cells of the younger primary and lateral roots. Knockdown of either

OsPT2 or *OsPT6* expression significantly decreased both the uptake and the long-distance transport of Pi from the roots to the shoots, suggesting that they are also involved in Pi uptake and translocation, but their relative contribution to the entire Pi uptake is unknown.

PHO1, belonging to a unique membrane protein family that is distinct from PHTs, has been implicated in the root-to-shoot translocation of Pi. In the rice genome, there are three PHO1 homologues. Interestingly, all of them have *cis*-natural antisense transcripts (*cis*-NAT). *OsPHO1;2* showed the most abundant expression in the roots (Secco et al. 2010). Knockout of OsPHO1;2 resulted in reduced Pi transfer from the roots to the shoots (Secco et al. 2010). A more recent study revealed that the *cis*-natural antisense transcript of PHO1;2 (*cis*-$NAT_{PHO1;2}$) has a function for promoting the translation of PHO1;2 and affecting phosphate homeostasis and finally plant fitness (Jabnoune et al. 2013).

Following xylem loading by PHO1, P is preferentially distributed by SPDT (SULTR-like phosphorus distribution transporter) at the nodes (Fig. 13.2). SPDT belongs to the sulfate transporter family but transports Pi rather than sulfate (Yamaji et al. 2017). It is highly expressed in the nodes, particularly in the uppermost node I at the reproductive stage. SPDT is localized at the xylem transfer cells and parenchyma cell bridge (PCB) and responsible for preferential distribution of P to developing new tissues and grains (Yamaji et al. 2017).

Potassium (K) plays an important role especially in regulating osmotic pressure. Transporters for K uptake have been identified in *Arabidopsis*. Two transporters, HAK5 (potassium transporter KUP/HAK/KT family) and AKT1 (ARABIDOPSIS K^+ TRANSPORTER1; shaker family potassium channel), have been reported to be involved in K^+ uptake (Hirsch et al. 1998; Gierth et al. 2005). However, little is known about transporters involved in K^+ uptake in rice. A homologue of *Arabidopsis* AKT1, OsAKT1, functions as an inward-rectifying channel and contributes to K^+ uptake (Li et al. 2014). Knockout of OsAKT1 resulted in decreased biomass and K^+ concentration of the roots and shoots at both vegetative and reproductive growth stages in the presence of low or normal K^+ (Li et al. 2014).

There are high- and low-affinity uptake systems for magnesium (Mg) (Tanoi et al. 2014). Recently, OsMGT1, a CorA-like gene encoding a member of the mitochondrial RNA splicing 2/magnesium transporter (MRS2/MGT) family in rice, was found to be involved in Mg uptake (Chen et al. 2012). OsMGT1 is localized at the plasma membrane. Knockout of this gene resulted in decreased Mg uptake (Chen et al. 2012). However, since Mg uptake in the *osmgt1* mutant is still remaining, it seems that other unidentified transporters are also responsible for Mg uptake from the soil solution to the root cells. In addition, OsMGT1 contributes to the alleviation of Al toxicity by increasing Mg concentration in the cell (Chen et al. 2012).

Sulfate (SO_4^{2-}) is the major form of sulfur (S) that plants can use for synthesizing sulfur-containing compounds, such as cysteine and methionine, proteins, lipids, coenzymes, and various secondary metabolites (Leustek et al. 2000; Saito 2004). Sulfate is absorbed by the SULTR transporter family members in plants. Extensive work has been done in *Arabidopsis* on SULTR transporters. There are

12 SULTR members in *Arabidopsis* (Takahashi et al. 2012a, b). In rice, there are 14 SULTR members showing different expression patterns (Kumar et al. 2011; Takahashi et al. 2012a). Among them, OsSULTR1;1, OsSULTR1;2, OsSULTR2;1, and OsSULTR5;2 are highly expressed in the seedling roots based on microarray analysis (Kumar et al. 2011). Moreover, most OsSULTR genes are up- or downregulated by several biotic and abiotic stresses (Kumar et al. 2011). Based on tissue expression, OsSULTR1;1 has been implicated in S uptake in rice roots (Godwin et al. 2003). However, the functions and physiological roles of most S transporter genes in rice have not yet been characterized. Following uptake of sulfate from the soil, it is loaded into xylem for subsequent translocation to aerial parts. However transporters involved in these steps are still unclear.

Transporters for calcium (Ca) are poorly understood. Some transporters belonging to the cation/H^+ exchanger family protein showed Ca^{2+} transport activity when they were expressed in yeast (Kamiya et al. 2005). However, the contribution of these transporters to Ca transport in rice remains to be determined because phenotypic analysis using knockout/knockdown of OsCAXs has not yet been investigated.

13.2.2 Transporters for Micronutrients

Manganese (Mn) is involved in many biological processes, such as water splitting activity in photosystem II, elimination of superoxide, and various secondary metabolisms. Plants require 50–100 mg kg^{-1} of Mn for their growth (Vlamis and Williams 1964). However, rice is able to accumulate Mn up to 5000 mg kg^{-1} DW without showing toxicity symptoms. It is now clear that this high Mn uptake is cooperatively mediated by an influx transporter (OsNramp5) and an efflux transporter (OsMTP9). *OsNramp5*, a member of the NRAMP (natural resistance-associated macrophage proteins) family, is mainly expressed in the root through all its growth stages (Sasaki et al. 2012). Furthermore, *OsNramp5* is highly expressed in the mature zone of the root, and its expression is not affected by Mn availability. It is a plasma membrane-localized influx transporter for Mn. OsNramp5 protein is polarly localized at the distal side of both the exodermis and endodermis (Sasaki et al. 2012, Fig. 13.1). In the knockout mutant of OsNramp5, the Mn uptake ability is almost lost (Sasaki et al. 2012), indicating that OsNramp5 is a major transporter for Mn uptake in rice (Ishikawa et al. 2012; Ishimaru et al. 2012; Sasaki et al. 2012), which is responsible for transporting Mn from the soil solution into the root cells. On the other hand, OsMTP9 (metal tolerance protein 9) belongs to the cation diffusion facilitator (CDF) family. It is a plasma membrane-localized efflux transporter for Mn (Ueno et al. 2015). Similar to *OsNramp5*, *OsMTP9* is also mainly expressed in the mature root zones. OsMTP9 protein is also localized to exodermis and endodermis cells, but, in contrast to OsNramp5, it is polarly localized at the proximal side of both cell layers (Ueno et al. 2015, Fig. 13.1). Knockout of OsMTP9 significantly decreased Mn uptake and root-

to-shoot translocation. These results indicated that the efficient Mn uptake in rice requires both OsNramp5 and OsMTP9.

A transporter belonging to the NRAMP family, OsNramp3, plays an important part in distributing Mn to different tissues in response to environmental Mn changes (Yamaji et al. 2013a). OsNramp3 is a plasma membrane-localized transporter of Mn localized in both xylem transfer cells of EVBs and phloem region of DVBs at both basal nodes at vegetative growth stage and upper nodes at reproductive growth stage (Fig. 13.2). Under Mn-limited condition, OsNramp3 at the transfer cells mediates unloading of Mn from the xylem of EVBs, followed by reloading Mn to the phloem of DVBs in nodes, delivering limited Mn preferentially to the developing new leaves and panicles. However, under excess Mn conditions, OsNramp3 protein was rapidly degraded, and the distribution of Mn follows the transpiration rate (Yamaji and Ma 2014).

Zinc (Zn) plays a key role in plants as an enzyme cofactor and a structural component of protein mediating binding to other proteins, DNA, RNA, or small molecules. Two members belonging to the ZIP (zinc-regulated transporters, iron-regulated transporter-like protein) family, OsZIP1 and OsZIP3, were suggested to be responsible for Zn uptake and/or homeostasis in rice (Ramesh et al. 2003; Bashir et al. 2012). However, recent work showed that OsZIP3 localized to the node is involved in the distribution of Zn to the developing tissues, rather than uptake (Sasaki et al. 2015). OsZIP1 is expressed in the epidermis and vascular tissues of roots and leaves (Ramesh et al. 2003, Bashir et al. 2012). Therefore, OsZIP1 might be involved in the uptake of Zn, but more physiological evidence is required for supporting this hypothesis.

Most of the Zn taken up by the roots is preferentially distributed to the nodes and developing organs but is not allocated to developed leaves (Obata and Kitagishi 1980a, b; Obata et al. 1980; Suzuki et al. 2008). The nodes accumulate Zn at very high level that is more than ten times higher than that of other tissues (Kitagishi and Obata 1986; Yamaguchi et al. 2012; Moore et al. 2014). For efficient distribution of Zn, two transporters localized at the node were identified. One is OsZIP3 described above, and the other is OsHMA2 (Sasaki et al. 2015; Yamaji et al. 2013b). OsZIP3 is localized at the xylem transfer cells in EVB and is involved in unloading of Zn from the xylem of EVB (Sasaki et al. 2015, Fig. 13.2). On the other, OsHMA2 is localized at the phloem region of both EVBs and DVBs and responsible for loading Zn to the phloem of DVBs and EVBs (Yamaji et al. 2013b, Fig. 13.2).

Iron (Fe) is essential for many cellular functions in plants, including chlorophyll biosynthesis, photosynthesis, and respiration. There are two strategies for iron (Fe) acquisition from soil depending on the plant species. Dicots and non-gramineous monocots adopt Strategy I, which is characterized by the reduction of ferric Fe (Fe^{3+}) to ferrous Fe (Fe^{2+}) by ferric chelate reductase (FRO) on the plasma membrane and subsequent uptake by the IRT1 transporter (Eide et al. 1996; Robinson et al. 1999). By contrast, gramineous plants adopt Strategy II, which is characterized by the secretion of mugineic acid (MA) and subsequent uptake of the mugineic acid–ferric complex (Ma and Nomoto 1996; Murata et al. 2006). However, rice seems to harbor both Strategy I and II. Although rice does not have

effective ferric chelate reductases in the roots (Ishimaru et al. 2006), ferrous Fe is rich in paddy fields due to reductive status in flooded paddy soil. Ferrous Fe was suggested to be taken up by OsIRT1 and OsIRT2, two members of the ZIP transporter family (Bughio et al. 2002; Ishimaru et al. 2006). Both OsIRT1 and OsIRT2 show a transport activity for ferrous Fe (Ishimaru et al. 2006). *OsIRT1* is expressed in the root epidermis and stele, and its expression is upregulated by Fe deficiency (Ishimaru et al. 2006). However, the contribution of these transporters to Fe uptake remains to be determined because phenotypic analysis using knockout/knockdown of OsIRT1 and OsIRT2 has not yet been performed.

On the other hand, rice is also able to take up the MA–Fe complex, which is mediated by OsYSL15, a member belonging to the yellow stripe-like family (Inoue et al. 2009; Lee et al. 2009). OsYSL15 is localized to the plasma membrane and shows transport activity for the MA–Fe complex (Inoue et al. 2009; Lee et al. 2009). *OsYSL15* in roots is strongly induced by Fe deficiency, but not by Zn, Mn, or Cu deficiency (Inoue et al. 2009). *OsYSL15* is expressed in the epidermis, endodermis, cortex, and vascular bundles of the roots as well as in the leaves under Fe deficiency condition (Inoue et al. 2009; Lee et al. 2009). Knockout of *OsYSL15* decreased the Fe concentration in both the shoots and roots (Lee et al. 2009). However, the contribution of this transporter in paddy soil would be small because secreted MA will be diffused out of the rhizosphere.

After Fe uptake, a citrate transporter, OsFRDL1, is involved in the translocation of Fe from the roots to the shoots (Yokosho et al. 2009). OsFRDL1 is localized at the plasma membrane of root pericycle cells. Knockout of this gene resulted in increased Fe accumulation in the roots, but decreased Fe in the shoots. On the other hand, OsYSL2 is implicated in long-distance transport of NA-chelated Fe into sink tissues (Ishimaru et al. 2010).

Boron (B) is essential for the formation of cross-links between polysaccharides in the cell walls. Two transporters (AtNIP5;1 and AtBOR1) for B uptake have previously been identified in *Arabidopsis* (Takano et al. 2002, 2006). A homologue of *Arabidopsis* B uptake transporter AtBOR1 in rice, OsBOR1, was also reported to be involved in B uptake as well as in xylem loading (Nakagawa et al. 2007). OsBOR1 is expressed in the root exodermis, endodermis cells, and the stele (Nakagawa et al. 2007). Knockout of this gene resulted in decreased B levels in the shoot and xylem sap. OsBOR1 is an efflux transporter for B; therefore, an influx transporter-like AtNIP5;1 is required for transporting B from the soil solution to the root cells in rice. OsNIP3;1, the closest homologue of AtNIP5;1, is also localized at the plasma membrane and is permeable to boric acid. It is expressed in both roots and shoots (Hanaoka et al. 2014), but knockdown lines of *OsNIP3;1* did not significantly affect B uptake (Hanaoka et al. 2014). Recently, however, *OsNIP3;1* was identified as a gene responsible for the dwarf phenotype observed in rice mutant *dte1* (*Dwarf and tiller-enhancing 1*) and was implicated in B uptake (Liu et al. 2015). *OsNIP3;1*/*DTE1* is expressed abundantly in the exodermal cells in the roots as well as in the nodal region of adult leaves. Furthermore, the expression was induced by B deficiency (Liu et al. 2015). Although the shoot B concentration in the *dte1* mutant was not changed from the WT, growth and total B content were

significantly decreased in the mutant (Liu et al. 2015). Therefore, OsNIP3;1/DTE1 and OsBOR1 may constitute a cooperative uptake system like Lsi1–Lsi2 for silicon (Si) uptake described below, although further investigation is required particularly in terms of protein localization in the roots.

Copper (Cu), as a cofactor in proteins, is involved in a wide variety of physiological processes. Cu uptake in the roots is likely mediated by a member of copper transporter (COPT), a homologue of the yeast Cu transporter CTR1 (Sancenón et al. 2003; Yuan et al. 2011). There are seven members of COPT1-like transporters in rice; some of them are expressed in the roots, and their expression is induced by Cu deficiency (Yuan et al. 2011). These COPT1-like transporters can transport Cu in Cu^{+} form. However it is still unclear which of these COPT-like transporters are involved in Cu uptake in rice roots. On the other hand, OsHMA5, a member of heavy metal-transporting P-type ATPase (HMA), mediates the xylem loading of Cu (Deng et al. 2013). OsHMA5 is mainly localized at the pericycle, and knockout of this gene resulted in decreased root-to-shoot translocation of Cu (Deng et al. 2013, Fig. 13.1). OsHMA5 is also localized at the xylem region of DVBs in node I (Deng et al. 2013, Fig. 13.2). It is likely that OsHMA5 also plays an important role in releasing Cu from these tissues into the xylem connected to the grains (Deng et al. 2013). Its homologue, OsHMA4, has been involved in Cu accumulation in grain (Huang et al. 2016). OsHMA4 is also a Cu transporter but localized to the tonoplast. It is highly expressed in the vascular tissues of the stele, mainly in pericycle cells in the roots (Fig. 13.1). The mRNA expression of *OsHMA4* is induced by high Cu treatment. Knockout of OsHMA4 resulted in lower Cu accumulation in roots but higher accumulation in the shoots, indicating that OsHMA4 functions to sequester Cu into root vacuoles, limiting Cu accumulation in the grain at high Cu condition (Huang et al. 2016).

Another transporter involved in the Cu transport is OsYSL16, which is a transporter belonging to the yellow stripe-like family (Zheng et al. 2012). OsYSL16 is expressed in phloem regions of EVBs and DVBs in the nodes (Fig. 13.2). In addition, OsYSL16 is highly expressed in vascular tissues of the leaves (Zheng et al. 2012). Knockout of OsYSL16 resulted in decreased remobilization of Cu-nicotianamine complex from old leaves to new leaves and from the flag leaf to the panicle (Zheng et al. 2012). So OsYSL16 is involved in the delivery of Cu-nicotianamine complex to young tissues and grains by phloem transport.

13.3 Transporters for Beneficial Elements

Silicon (Si) is a beneficial element for plant growth, protecting plants from various stresses (Ma and Yamaji 2006, 2015). Plants take up Si from the soil in the form of silicic acid, a non-charged form below pH 9.0. Two transporters (Lsi1 and Lsi2) for Si uptake have been identified in rice (Ma et al. 2006, 2007). Lsi1 belongs to the Nod26-like major intrinsic protein (NIP) subfamily of aquaporins (Ma et al. 2006)

and is a Si-permeable channel. On the other hand, Lsi2, belonging to a putative anion transporter protein family, is an efflux transporter of Si (Ma et al. 2007). Both of them are localized at the root exodermis and endodermis with different polarity; Lsi1 is at the distal side and Lsi2 is at the proximal side (Fig. 13.1). Knockout of either Lsi1 or Lsi2 in rice results in a defect in Si uptake. Therefore, a cooperative transport by both Lsi1 and Lsi2 is required for the efficient uptake of Si in rice. Mathematical modeling revealed that the polar localization of Lsi1 and Lsi2 and the presence of Casparian strips are essential for efficient Si uptake in rice (Sakurai et al. 2015).

Following uptake by the roots through Lsi1 and Lsi2, Si is translocated to the shoot by transpirational mass flow in the xylem. In rice, more than 90% of Si taken up by the roots is translocated to the shoots (Ma and Takahashi 2002). Si in xylem of EVBs is unloaded by another Si transporter Lsi6 (Fig. 13.2), moved to adjacent cells through plasmodesmata, and then effluxed to the apoplast and reloaded to xylem of DVBs by Lsi2 and Lsi3, a homologue of Lsi2 (Yamaji and Ma 2009; Yamaji et al. 2008, 2015, Fig. 13.2). These three transporters are responsible for the preferential distribution of Si to the grain, which is required for high Si accumulation in the husk.

13.4 Transporters for Toxic Elements

Uptake of toxic elements such as cadmium (Cd) and arsenic (As) will affect our health through food chain. Rice is a major dietary source of Cd and As. Several transporters involved in Cd accumulation have been identified in rice. OsNramp5 described above as a Mn uptake transporter also transports Cd (Sasaki et al. 2012). A *Nramp5* knockout mutant and RNAi lines grown in soil show a strongly reduced Cd concentration of the straw and the grain. Other transporters such as OsIRT1, OsIRT2, and OsNramp1 (these are Fe^{2+} transporters induced under Fe deficiency) are potentially implicated in Cd uptake. However because OsNramp5 was shown to account for most of the rice Cd uptake under different Fe supply conditions, OsIRTs and OsNramp1 appear to play only a minor role in Cd uptake from the soil (Sasaki et al. 2012).

Part of Cd taken up is sequestered into vacuoles in the roots. This vacuolar sequestration process is facilitated by P_{1B}-type ATPase, OsHMA3 (heavy metal ATPase 3) (Ueno et al. 2010; Miyadate et al. 2011). OsHMA3 is localized at the tonoplast of all root cells (Fig. 13.1) and has an efflux transport activity of Cd. When OsHMA3 was overexpressed in rice, the transgenic plants greatly increased the Cd accumulation in the roots and strongly reduced Cd accumulation in the shoots (Sasaki et al. 2014). Another HMA family protein, OsHMA2, is responsible for the root-to-shoot translocation of Cd in rice (Satoh-Nagasawa et al. 2012; Takahashi et al. 2012b; Yamaji et al. 2013b). OsHMA2 is a transporter of Zn in rice and is localized in the plasma membrane of root pericycle cells at the vegetative growth stage (Fig. 13.1). Knocking out this transporter significantly

decreased the Zn concentration in the root tips and shoot elongating zone, suggesting that OsHMA2 transports Zn and Cd from the apoplast to the symplast to facilitate translocation via the phloem.

Low-affinity cation transporter 1 (OsLCT1) and OsHMA2 are involved in intervascular transfer of Cd in the nodes (Uraguchi et al. 2011; Yamaji et al. 2013b). The transcripts of *OsLCT1* were detected in cells surrounding the EVBs and DVBs and the phloem parenchyma cells of EVBs of node I. Knockdown of *OsLCT1* resulted in the reduction of grain Cd after grown in Cd-contaminated soil. The Cd concentration is reduced in the phloem sap but not in the xylem sap of the mutant. It seems that OsLCT1 plays a role in xylem-to-phloem transfer in node I.

OsHMA2 is also expressed in the upper nodes during the reproductive stage (Yamaji et al. 2013b). OsHMA2 was detected in the plasma membrane of phloem companion cells in EVBs and DVBs (Fig. 13.2). Mutation of OsHMA2 resulted in lower Cd accumulation in the tissues above node I, suggesting that OsHMA2 plays a role in the preferential delivery of Cd to these tissues.

On the other hand, As is a highly toxic metalloid for animals including human, and exposure to excessive As causes numerous diseases, such as cancers, diabetes, cardiovascular diseases, and developmental disorders (Abdul et al. 2015). A number of As species may be present in soil depending on soil conditions and the history of As contamination. These include arsenate [As(V)], arsenite [As(III)], and methylated As species such as monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)]. Paddy rice shows about ten times higher accumulation of As compared with other cereal crops (Williams et al. 2007). High accumulation of As in paddy rice is caused by the mobilization of As in flooded paddy soil (Xu et al. 2008) and the highly efficient uptake of As by the silicon (Si) pathway in rice (Ma et al. 2008). In the anaerobic paddy field, As is mainly present in the form of arsenite (As(III)), which is taken up by two Si transporters, namely, Lsi1 and Lsi2 (Ma et al. 2008), which have high expression in the roots (see above; Fig. 13.1). Not only Lsi1 and Lsi2 but also other transporters might be involved in the transport of As(III) (Li et al. 2016).

After As(III) is taken up by the root cells, a part of this is released from the cytosol to the rhizosphere by the bidirectional transport of Lsi1 (Zhao et al. 2010a, Fig. 13.1). However, the remaining As are sequestrated into the root vacuoles or translocated to the shoots (Zhao et al. 2010b). For the detoxification of As by vacuolar sequestration, the complexation of As(III) with phytochelatins (PCs) is important. Recently, a tonoplast-localized ABC transporter, OsABCC1, was found to be involved in the As(III)–PC sequestration into the vacuoles (Song et al. 2014). *OsABCC1* was expressed in many organs, including the roots, leaves, nodes, peduncle, and rachis (Figs. 13.1 and 13.2). Knockout of OsABCC1 in rice resulted in decreased tolerance to As. At the reproductive stage, the As concentration was higher in the nodes and in other tissues of wild-type rice than in those of knockout mutants but was significantly lower in the grain. Therefore, OsABCC1 restricts the translocation of As into grains by sequestering As into the vacuoles in the nodes.

13.5 Control Mechanisms for Mineral Transport: Perception, Signaling, and Responses

Availability of mineral elements for plants in natural environment varies greatly depending on the weather, soil, growth conditions, and so on. But plants have many strategies to overcome mineral unbalances. Transporters involved in the uptake and translocation respond to these environmental changes at transcriptional and/or translational levels. For example, the gene expressions of OsPT6 and OsPT8 were upregulated by P deficiency condition (Ai et al. 2009; Jia et al. 2011). Fe^{2+} transporters *OsIRT1* and *IRT2* were also highly upregulated in response to iron deficiency condition. Several transcription factors including IDE1 and IDE2 and OsIRO2 and OsIRO3, which control Fe deficiency-induced genes, have been identified in rice (Kobayashi and Nishizawa 2012). Recently, two iron- and zinc-binding RING ubiquitin ligases, OsHRZ1 and OsHRZ2, were suggested to be Fe-binding sensors (Kobayashi et al. 2013). Knockdown of these genes resulted in increased tolerance to low iron availability but inferior growth under extreme iron excess conditions. The knockdown lines showed derepressed expression of various genes involved in iron uptake and transport, mainly under enough or excess iron availability, indicating that HRZ ubiquitin ligases negatively regulate the expression of the genes involved in iron uptake, transport, and accumulation, inhibiting excess iron accumulation.

Mn in soil solution fluctuates greatly from 0.5 to 200 μM depending on soil conditions. The transporters involved in Mn uptake (OsNrmap5 and OsMTP9) did not respond to external Mn concentration, whereas OsNramp3 localized at the nodes responded to high Mn at the protein level. Under Mn-limited condition, OsNramp3 mediates preferential distribution of Mn to the developing tissues. However, under high Mn condition, this protein was degraded within few hours although the transcript level of *OsNramp3* is unchanged.

More detailed mechanisms for the regulation of mineral acquisition have been investigated for some transporters. For example, in rice, Pi transporters are regulated at the transcriptional and posttranslational levels (Bayle et al. 2011; Wu et al. 2013). Recently, a CK2 kinase was found to regulate the trafficking of Pi transporters in response to phosphate availability (Chen et al. 2015). In P-sufficient conditions, Pi transporters (OsPHT1;2/OsPT2 and OsPHT1;8/OsPT8) are phosphorylated by the CK2α3/β3 holoenzyme, resulting in an inhibition of the interaction of PT8 with PHF1(phosphate transporter traffic facilitator1), a key cofactor regulating the exit of PTs from the endoplasmic reticulum (ER) to the plasma membrane. By contrast, low-Pi stress leads to a reduction of the phosphorylation and abundance of CK2β3 which limits CK2α3 access to PTs for phosphorylation. Therefore, the non-phosphorylated PTs can interact with PHF1 for efficient trafficking from the ER to the PM (Chen et al. 2015). In *Arabidopsis*, it was found that the PHO2 ubiquitin-conjugating E2 enzyme modulates the degradation of PHO1 in the endomembranes to maintain Pi homeostasis (Liu et al. 2012), but it is not known whether similar mechanisms are present in rice.

Regulatory mechanism of nitrate uptake has been extensively investigated in *Arabidopsis*. First, nitrate is sensed by nitrate transporter 1.1 (NRT1.1), which is switched from low to high affinity due to the phosphorylation of threonine-101 (T101) (Ho et al. 2009). Under low-nitrate conditions, CBL-interacting protein kinase 23 (CIPK23) can phosphorylate T101, making NRT1.1 function as a high-affinity nitrate carrier and then leading to a weak induction of gene expression of *NRT2.1* (Ho et al. 2009). *NRT2.1* is a high-affinity nitrate transporter gene, which shows a rapid induction of gene expression in response to nitrate treatments independent of protein synthesis. But it is not known whether similar mechanisms are present in rice.

13.6 Perspective

During the last decade, several transporters involved in the uptake and distribution of mineral elements have been identified as described above (Figs. 13.1 and 13.2); however, most transporters remain to be identified in the future. Plants use different transporters for different mineral elements. For the same mineral element, different transporters are required for uptake, root-to-shoot translocation, and distribution/redistribution. Furthermore, due to the distinct root anatomy of rice, a pair of influx and efflux transporters is required for the radial transport of mineral elements from the soil to the stele in order to cross the two Casparian strips at both exodermis and endodermis. However, the exact roles of most transporters are unknown, especially in terms of tissue and cellular localization. The regulation of these transporters in response to changing environments (deficiency or excess of mineral elements) also remains to be examined. Further investigation of these transporters will not only help to better understand mineral transport system in rice but also provide new tools for breeding rice with high nutrient use efficiency and low accumulation of toxic mineral elements. On the other hand, large genotypic differences in mineral accumulation and use efficiency have been reported. Identification of new alleles by using these genotypic differences will also provide new tools for breeding.

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Chapter 14
Genetic Mechanisms Involved in the Formation of Root System Architecture

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Abstract Root system is essential for absorbing water and nutrients as well as anchoring shoots to the ground. Understanding the genetic mechanisms related to the formation of root system architecture is necessary for improving rice productivity. Here, we first describe the potential of genetic improvement using quantitative trait locus (QTL) for root system architecture based on our field experiments using a genetic material of *DEEPER ROOTING 1*, which is a rice QTL controlling root growth angle. Next, we summarize the accumulated knowledge on the genetic mechanisms of root formation in rice including the development of the radicle, crown roots, lateral roots, and root hairs. We also overview the current status of the genetic dissection of root system architecture in rice, namely, the identification and characterization of natural and artificial alleles. Root traits are rarely chosen as breeding targets because their evaluation in a large number of plants under field conditions is more laborious and time-consuming than evaluation of aboveground traits. The genetic dissection of root system architecture would facilitate the breeding of root traits, eventually improving rice yield irrespective of soil and other environmental conditions.

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14.1 Introduction

14.1.1 What Is the Ideotype for the Second Green Revolution?

Half a century ago, ideotype breeding using a semidwarf gene, *sd1*, resulted in the Green Revolution in rice (Khush 2001), but breeders and researchers still have to increase rice yield because rice is a staple food for nearly half of the world's population. Thus, we need to consider what the ideotype for the second Green Revolution could be. Recent climate change is increasing the inequality of water and nutrient distribution in agricultural lands at a global level; global warming has caused serious drought damage in farmlands that rely on rainwater or that have limited access to irrigation (Scheffran and Battaglini 2011). What kinds of traits are needed to improve rice yield in this situation?

Root system traits should be such candidate traits to achieve the second Green Revolution. Root, which is the only organ absorbing water and nutrients from soil, is imperative for the survival of terrestrial plants, which cannot move around after germination. Optimal root development and distribution allow efficient acquisition of water and nutrients, which are heterogeneously distributed in soil (Gowda et al. 2011; Lynch 2013). For example, the topsoil tends to hold less water but more immobile nutrients such as phosphorus than does the subsoil. Nitrate, which is the main form of nitrogen under aerobic conditions, is leached by precipitation into subsoil. Therefore, root system architecture greatly affects the acquisition of water and nutrients from soil (Gewin 2010; Lynch 1995). Many wild species tend to have adequate root systems to adapt to severe stresses (Canadell et al. 1996); for example, drought-resistant plants tend to develop deeper root systems, which allow them to capture water from subsoil and thus avoid drought stress. Increased roots in subsoil would be also effective to avoid the negative impacts of drought on crop yield (de Dorlodot et al. 2007). Therefore, the genetic improvement of root system architecture has been regarded as an important approach to enhance crop production. However, it is more laborious and time-consuming to select root traits than aboveground traits. Therefore, many researchers are considering molecular breeding by using quantitative trait loci (QTLs) as one of the promising strategies for improving root system architecture (de Dorlodot et al. 2007; Yamamoto et al. 2014).

14.1.2 Prospects of Ideotype Breeding Using QTLs for Root System Architecture

In rice, a wide natural variation of root system architecture has been reported (Lafitte et al. 2001; O'Toole and Bland 1987; Uga et al. 2009). For example, typical upland rice has deeper, longer, and thicker roots than lowland rice (O'Toole and Bland 1987) and thus might be a useful breeding material to improve rice productivity. Root system architecture is a complex trait controlled by tens to hundreds of genes (Wachsman et al. 2015). Courtois et al. (2009) summarized 675 rice QTLs for 29 root parameters detected in 12 mapping populations reported in 24 published papers, but only one QTL associated with root system architecture has been isolated as a single gene in rice (Uga et al. 2013a).

The rice QTL *DEEPER ROOTING 1* (*DRO1*) has been identified on chromosome 9 in recombinant inbred lines derived from a cross between the lowland cultivar "IR64" and the upland cultivar "Kinandang Patong" (Uga et al. 2011) and has been cloned (Uga et al. 2013a). "Kinandang Patong," which has a functional allele of *DRO1*, has deep roots, whereas "IR64," which has a nonfunctional allele, has shallow roots. In a near-isogenic line (Dro1-NIL) that carries *DRO1* derived from "Kinandang Patong" in the genetic background of "IR64," *DRO1* increases root growth angle, resulting in deep rooting, but has a limited effect on other root and shoot traits. Field experiments using this unique line, which differs from "IR64" only in the increased root growth angle, have demonstrated that alteration of root system architecture improves rice productivity, as discussed in detail below (Fig. 14.1).

The yield performance of "IR64" and Dro1-NIL was compared under upland field conditions with no drought, moderate drought, or severe drought (Uga et al. 2013a). Under moderate drought in comparison with no drought, the grain weight of "IR64" decreased by nearly half, whereas that of Dro1-NIL was almost the same. Under severe drought, the grain weight of "IR64" was very low, whereas that of Dro1-NIL was more than 30% of that with no drought. This study suggests that deep rooting induced by *DRO1* enhances drought avoidance, resulting in higher grain yield (Fig. 14.1a).

Comparison among cultivars with different root and shoot morphologies has suggested that deep roots increase grain yield in paddy fields (Kawata et al. 1978; Morita et al. 1988), but the genetic aspects of this effect have not yet been clear in previous studies. In paddy fields, Dro1-NIL showed about 10% higher grain yield than did "IR64," irrespective of nitrogen treatment (Arai-Sanoh et al. 2014). There was no significant difference between "IR64" and Dro1-NIL in nitrogen content before heading, but nitrogen uptake was higher in Dro1-NIL than in "IR64" after heading. These results suggest that deep rooting induced by *DRO1* enhances nitrogen uptake from lower soil layers, resulting in better grain filling (Fig. 14.1b).

Because root growth angle influences the efficiency of nitrogen absorption, it might also affect the uptake of other minerals such as heavy metals. In Cd-contaminated soil, the grain and straw Cd concentrations were significantly

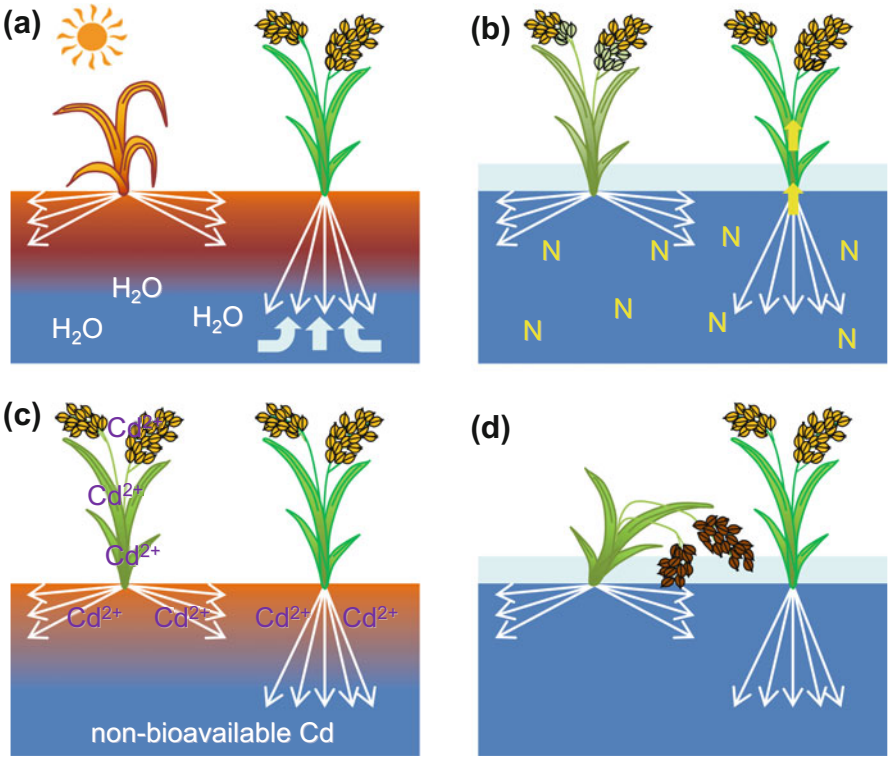

Fig. 14.1 Schematic models of the effect of *DRO1* on rice production and phytoremediation. (**a**) Drought avoidance (water uptake). In upland fields, where water is most abundant in subsoil, deep rooting caused by *DRO1* allows plants to efficiently capture water. (**b**) Yield performance (nitrogen uptake). Under irrigated conditions in paddy fields, deep rooting caused by *DRO1* allows plants to access nitrogen from the subsoil during reproductive stages. (**c**) Phytoremediation (Cd uptake). In a rain-fed paddy field after drainage, the bioavailable Cd concentration increases in the topsoil. Shallow rooting caused by *dro1* allows plants to accumulate bioavailable Cd from the topsoil. (**d**) Lodging resistance. In wet paddy fields, deep rooting caused by *DRO1* increases pushing resistance (an index of lodging resistance). Rice plants described in each part are IR64 on the left and Dro1-NIL on the right

higher in "IR64" than in Dro1-NIL (Uga et al. 2015a). These results were opposite to those in the case of nitrogen uptake because the bioavailable Cd concentration was increased in the oxidized topsoil layer by withholding irrigation water during the vegetative growth period. Therefore, shallow roots could capture Cd from topsoil, resulting in a high concentration of Cd in rice plants (Fig. 14.1c). This suggests that, for phytoremediation, the allele occurring shallow rooting is a potential genetic resource for developing plants with high Cd accumulation. From the viewpoint of food safety, the allele giving deep rooting could be a useful resource to avoid absorbing the bioavailable Cd from topsoil.

Comparisons of different cultivars and examination of the effects of root pruning suggest that deep roots influence lodging resistance (Sakata et al. 2004; Terashima 1997; Terashima et al. 1994, 1995), although the genetic aspects of this effect are still unknown. Under wet paddy field conditions, Dro1-NIL had stronger pushing resistance (an index of lodging resistance) than "IR64" (Arai-Sanoh et al. 2014), suggesting that deep rooting induced by *DRO1* improves lodging resistance (Fig. 14.1d).

These field experiments with Dro1-NIL confirm the potential of ideotype breeding for root system architecture, although further studies in other environments are needed. To design new root ideotypes that are adapted to diverse environmental stresses and to conduct ideotype breeding by using marker-assisted selection (Coudert et al. 2010), it will be necessary to update our understanding of the genetic mechanism associated with root system architecture. Information on gene networks involved in root formation has been accumulated for the model dicot plant *Arabidopsis thaliana*, but our knowledge of these aspects in rice is limited (Coudert et al. 2010; Rebouillat et al. 2009). In this chapter, we overview this knowledge and discuss the prospects of applying it to molecular breeding.

14.2 Root Formation

The first root of a rice plant, the radicle, is generated during embryogenesis. A radicle primordium originates endogenously from the embryo 4 days after pollination (DAP) (Itoh et al. 2005), whereas in *Arabidopsis* it is exogenously differentiated from the hypophysis (Dolan et al. 1993). After germination, the radicle is named the seminal root. Crown roots originate from the parenchyma cells adjacent to the peripheral cylinder of vascular bundles of the stem; therefore, crown roots are also named shoot-born roots (Fig. 14.2). Monocots develop a fibrous root system characterized by numerous crown roots, meanwhile dicots consist of only a main root system (Klepper 1992). A rice plant usually generates several hundreds of crown roots under field conditions (Klepper 1992). Lateral roots grow from seminal and crown roots (Fig. 14.2) and are responsible for taking up most water absorbed by the root system (Varney et al. 1993). In rice, two distinct types of lateral roots have been identified (Kawata and Shibayama 1965). L-type lateral roots are generally long and thick and are able to generate higher-order lateral branches, whereas S-type lateral roots are short, slender, and non-branching. Crown and lateral roots are classified as postembryonic roots because they are initiated after embryogenesis, whereas the seminal root (radicle) is generated during embryogenesis. Root hairs are tubular outgrowths of some root epidermal cells. Root hairs occupy most of the root surface area; they are thought to be important for water and nutrient uptake, anchorage, and interactions with soil microbes (Kim et al. 2007). Identification of genes associated with the formation of the different types of roots in rice has progressed together with the advances in molecular biology and DNA sequencing technology (Table 14.1).

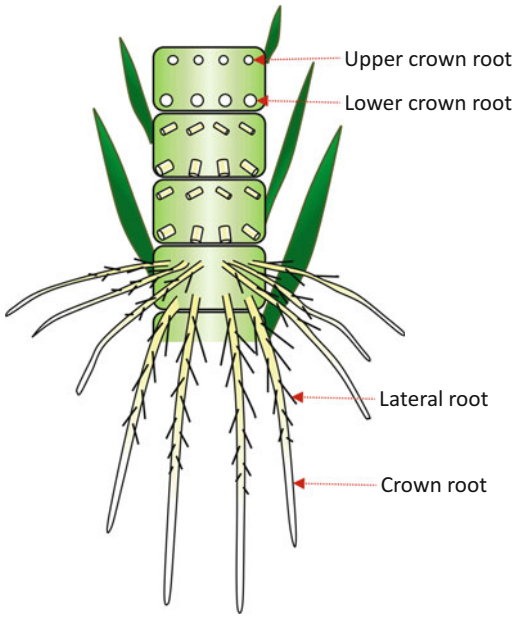

Fig. 14.2 Schematic view of the fibrous root system in rice

14.2.1 Root Apical Meristem

Except root hairs, each root type has a multicellular organization that can be described in terms of proximal-distal and radial polarity. All root cells are generated from stem cell daughters in the root apical meristem (RAM). Coordinated balance between cell division and differentiation is observed along the proximal-distal axis in the root tip, which can be divided into three zones according to cell division and elongation status: proximal division zone, transition zone, and distal elongation zone (Dolan et al. 1993). In rice, the root tip is formed by different types of cells arranged in concentric layers. The stele consists of the metaxylem, phloem, fibers, and pericycle and is surrounded by the endodermis, cortex, sclerenchyma, exodermis, and epidermis. In rice, these five cell layers are generated from single epidermis-endodermis structural initial cells by eight successive asymmetrical periclinal cell divisions following the first anticlinal division (Rebouillat et al. 2009).

The central region of RAM contains mitotically inactive cells, or the quiescent center (QC). The QC region in rice was examined by in situ hybridization with a probe for the rice *CYCLIN-DEPENDENT KINASE* (*CDK*) gene, a marker of cell division (Umeda et al. 1999). The analysis suggested that rice QC is large, unlike that of *Arabidopsis*, which consists of only four cells. In rice, the expression of a *WUSCHEL* (*WUS*)-type homeobox gene designated *QUIESCENT-CENTER-SPECIFIC HOMEOBOX* (*QHB*) was detected in the central cells of QC, similar to the

Table 14.1 Root development-related genes in rice

Phenotype	Gene	Chromosome	Locus ID		Encoded protein	References
			RAP	MSU		
Crown root number	*FIB*/*OsTAA1*	1	Os01g0169800	LOC_Os01g07500	Aminotransferase	Yoshikawa et al. (2014)
	OsRAA1	1	Os01g0257300	LOC_Os01g15340	GTP-binding protein	Ge et al. (2004)
	OsPIN3t (*OsPIN10a*)	1	Os01g0643300	LOC_Os01g45550	Auxin efflux carrier	Zhang et al. (2012a)
	OsYUC1	1	Os01g0645400	LOC_Os01g45760	Flavin monooxygenase	Yamamoto et al. (2007)
	ERF3	1	Os01g0797600	LOC_Os01g58420	AP2/ERF transcription factor	Zhao et al. (2015a)
	OsCKX4	1	Os01g0940000	LOC_Os01g71310	Cytokinin oxidase/dehydrogenase	Gao et al. (2014)
	OsCAND1	2	Os02g0167700	LOC_Os02g07120	SCF^{TIR1} ubiquitin ligase	Wang et al. (2011)
	OsRR2	2	Os02g0557800	LOC_Os02g35180	Type-A response regulator	Zhao et al. (2009)
	REH1/ *OsPIN1b*	2	Os02g0743400	LOC_Os02g50960	Auxin efflux carrier	Xu et al. (2005)
	CRL1/*ARL1*	3	Os03g0149100	LOC_Os03g05510	LOB/ASL transcription factor	Inukai et al. 2005), Liu et al. (2005)
	OsCOW1 (*OsYUC8*)	3	Os03g0162000	LOC_Os03g06654	Flavin monooxygenase	Woo et al. (2007)
	CRL4/ *OsGNOM1*	3	Os03g0666100	LOC_Os03g46330	Arf-GEF	Kitomi et al. (2008b), Liu et al. (2009)
	OsRR1	4	Os04g0442300	LOC_Os04g36070	Type-A response regulator	Kitomi et al. (2011)
	OsMT2b	5	Os05g0111300	LOC_Os05g02070	Metallothionein	Yuan et al. (2008)
	OsPIN2	6	Os06g0660200	LOC_Os06g44970	Auxin efflux carrier	Chen et al. (2012b)
	CRL5	7	Os07g0124700	LOC_Os07g03250	AP2/ERF transcription factor	Kitomi et al. (2011)
	CRL6	7	Os07g0497100	LOC_Os07g31450	CHD family protein	Wang et al. (2016)
	WOX11	7	Os07g0684900	LOC_Os07g48560	WUS-related homeobox protein	Zhao et al. (2009)
	OsIAA3 (*OsIAA31*)	12	Os12g0601400	LOC_Os12g40900	Aux/IAA protein	Nakamura et al. (2006)

(continued)

Table 14.1 (continued)

Phenotype	Gene	Chromosome	Locus ID		Encoded protein	References
			RAP	MSU		
	OsPID	12	Os12g0614600	LOC_Os12g42020	Serine/threonine kinase	Morita and Kyozuka 2007)
	OsmiR393a	1	n.a.	n.a.	MicroRNA	Bian et al. (2012)
	OsmiR393b	4	n.a.	n.a.	MicroRNA	Bian et al. (2012)
Lateral root number	*OsAUX1*	1	Os01g0856500	LOC_Os01g63770	Auxin influx carrier	Zhao et al. (2015b)
	LRL2/ *OsCYP2*	2	Os02g0121300	LOC_Os02g02890	Cyclophilin	Kang et al. (2013)
	OsIAA11	3	Os03g0633500	LOC_Os03g43400	Aux/IAA protein	Zhu et al. (2012)
	OsIAA13	3	Os03g0742900	LOC_Os03g53150	Aux/IAA protein	Kitomi et al. (2012)
	OsHO1	3	Os06g0603000	LOC_Os06g40080	Heme oxygenase	Chen et al. (2012a)
	OsORC3	10	Os10g0402200	LOC_Os10g26280	Origin recognition complex protein	Chen et al. (2013)
	OsWOX3A/ *OsNS* (*NAL2*)	11	Os11g0102100	LOC_Os11g01130	WUS-related homeobox protein	Cho et al. (2013)
	OsWOX3A/ *OsNS* (*NAL3*)	12	Os12g0101600	LOC_Os12g01120	WUS-related homeobox protein	Cho et al. (2013)
Root hair number	*OsFH1*	1	Os01g0897700	LOC_Os01g67240	Multidomain protein	Huang et al. (2013a)
	SRH2	3	Os03g0300000	LOC_Os03g18820	Xyloglucan 6-xylosytransferase	Wang et al. (2014a)
	OsEXPA17	6	Os06g0108600	LOC_Os06g01920	Expansin	Yu et al. (2011)
	OsRHL1	6	Os06g0184000	LOC_Os06g08500	bHLH transcription factor	Ding et al. (2009)
	RTH1/ *OsAPY1*	7	Os07g0682800	LOC_Os07g48430	ATP diphosphohydrolase	Yuo et al. (2009)
	OsSNDP1	10	Os10g0122600	LOC_Os10g03400	Phosphatidylinositol transfer protein	Huang et al. (2013b)
	OsEXPA30	10	Os10g0535900	LOC_Os10g39110	Expansin	Yu et al. (2011)
	RTH2/ *OsCSLD1*	10	Os10g0578200	LOC_Os10g42750	Cellulose synthase-like protein	Kim et al. (2007), Yuo et al. (2011)

Root meristem formation	*QHB*	1	Os01g0854500	LOC_Os01g63510	WUSCHEL-type homeobox protein	Kamiya et al. (2003b)
	DOCS1	2	Os02g0236100	LOC_Os02g14120	LRR-RLK	Huang et al. (2012)
	OsIAA23	6	Os06g0597000	LOC_Os06g39590	Aux/IAA protein	Ni et al. (2011)
	OsSHR1	7	Os07g0586900	LOC_Os07g39820	GRAS transcription factor	Kamiya et al. (2003a), Cui et al. (2007)
	OsSCR1	11	Os11g0124300	LOC_Os11g03110	GRAS transcription factor	Kamiya et al. (2003a), Cui et al. (2007)
Root length	*OsEXPA8*	1	Os01g0248900	LOC_Os01g14650	Expansin	Ma et al. (2013)
	OsSPR1	1	Os01g0898300	LOC_Os01g67290	Armadillo-like repeat domain protein	Jia et al. (2011)
	OsMOGS	1	Os01g0921200	LOC_Os01g69210	Mannosyl-oligosaccharide glucosidase	Wang et al. (2014b)
	OsCYT-INV1	2	Os02g0550600	LOC_Os02g34560	Alkaline/neutral invertase	Jia et al. (2008)
	OsCKI1	2	Os02g0622100	LOC_Os02g40860	Putative casein kinase I	Liu et al. (2003)
	RT/*OsGLU3*	4	Os04g0497200	LOC_Os04g41970	Endo-1,4-β-D-glucanase	Inukai et al. (2012), Zhang et al. (2012b)
	OsARF12	4	Os04g0671900	LOC_Os04g57610	Auxin response factor	Qi et al. (2012)
	OsRPK1	5	Os05g0486100	LOC_Os05g40770	LRR-RLK	Zou et al. (2014)
	OsDGL1	7	Os07g0209000	LOC_Os07g10830	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase	Qin et al. (2013)
	OsGNA1	9	Os09g0488000	LOC_Os09g31310	Glucosamine-6-P acetyltransferase	Jiang et al. (2005)

n.a. not available

expression of its *Arabidopsis* ortholog *WUS-RELATED HOMEOBOX 5* (*WOX5*). *WOX5* is involved in QC maintenance, suggesting that *QHB* plays a similar role in rice (Kamiya et al. 2003b; Sarkar et al. 2007). The phytohormone auxin is crucial for QC maintenance (Friml et al. 2002; Sabatini et al. 1999). A mutation in a member of the *AUXIN* (*Aux*)/*INDOLE-3-ACETIC ACID* (*IAA*) gene family, *OsIAA23*, causes defects in postembryonic QC maintenance due to the disintegration of the root cap and termination of root growth, suggesting the importance of auxin in rice QC maintenance (Ni et al. 2011). The GRAS family genes *SCARECROW* (*SCR*) and *SHORT-ROOT* (*SHR*) are also the key regulators of QC maintenance and root radial patterning (Di Laurenzio et al. 1996; Helariutta et al. 2000; Sabatini et al. 2003). *OsSCR1* is specifically expressed in the endodermis, whereas *OsSHR1* is expressed in the stele, similar to the patterns of their expression in *Arabidopsis*; OsSCR1 and OsSHR1 interact with each other when produced in yeast, similar to SCR and SHR in *Arabidopsis* (Cui et al. 2007; Kamiya et al. 2003a). These data suggest that *OsSCR1* and *OsSHR1* control the division of the epidermis-endodermis initial cells in rice. Concerning the outer cell layers (epidermis, exodermis, and sclerenchyma) in rice, a mutation in *DEFECTIVE IN OUTER CELL LAYER SPECIFICATION 1* (*DOCS1*), which encodes a leucine-rich repeat receptor-like kinase (LRR-RLK), causes irregular epidermal cells with far fewer root hairs and transformation of some exodermal cells into additional sclerenchyma-like cells (Huang et al. 2012). The outer cell layers play an important role in protecting the inner root tissues from various stresses (Huang et al. 2009). Proper development of both inner and outer cell layers is essential for root development.

14.2.2 Radicle

A fertilized egg (zygote) undergoes iterative cell divisions to form a globular embryo with no apparent morphological differentiation until 3 DAP, and a radicle primordium is observed at 4 DAP together with a shoot apical meristem (Itoh et al. 2005). Therefore, radicle initiation is assumed to occur at the globular stage. The molecular mechanisms of radicle formation in rice and the key genes involved are presumed on the basis of experimental reports in *Arabidopsis*. Some rice mutants have defects in radicle formation; however, the causative genes have not yet been isolated.

Hong et al. (1995) reported three independent lines of *radicleless* (*ral*) mutants. One of them, *ral1*, is viable, although it has a reduced number of crown and lateral roots (Scarpella et al. 2003). The *ral1* plants also have narrower leaves with vascular patterning distortions associated with a defective response to auxin, indicating that *RAL1* has auxin-related functions. A mutant of *Oryza sativa CONNECTED EMBRYO* (*OsCEM*) produces multiple shoots and radicles (Yang and Hwa 2008). Endogenous indole-3-acetic acid (IAA) level in *oscem* embryos is lower than that in wild-type embryos despite no differences in vegetative stages.

This result also strongly suggests the relationship between auxin and radicle formation. Multiple radicles are sometimes observed in the *apical displacement 1* (*apd1*) mutant, in which the apical shoot region and scutellum are reduced and the basal region of the radicle is enlarged (Kinae et al. 2005). Formation of multiple radicles in *apd1* may be a secondary effect of the aberrant apical-basal patterning of the embryo (Kinae et al. 2005).

Our knowledge of radicle formation is limited compared to that of other types of rice roots because there are few reports of radicle-related genes. Recently, microarray analysis of spatiotemporal gene expression patterns during early embryogenesis was performed (Itoh et al. 2016). Based on the datasets from that study, we listed putative radicle-related genes and assessed their functions by performing in situ hybridization (Fig. 14.3). The list contained 23 genes (including 14 encoding transcription factors), which were classified into three groups according to their expression patterns (Fig. 14.3): genes with radicle-specific expression from radicle initiation to its development (Group I, 14 genes); genes with radicle-specific expression during radicle development (Group II, 4 genes); and genes not showing radicle-specific expression (Group III, 5 genes). We then focused on the transcription factor genes in Group I (Fig. 14.3a). Four genes for APETALA 2 (AP2)/ETHYLENE RESPONSIVE FACTOR (ERF) transcription factors with two AP2 repeats (Os06g0657500, Os02g0614300, Os04g0504500, and Os01g0899800) were expressed in the center of the 3-DAP embryo where a radicle will be formed (Fig. 14.3a). They share sequence similarities with *PLETHORA* (*PLT*) homologs, the key factors in root meristem formation and maintenance (Aida et al. 2004; Galinha et al. 2007). They are assumed to have roles in radicle formation, especially meristem initiation and development because PLT protein dosage in RAM, which is determined by auxin, is translated into distinct cellular responses: high levels of PLT promote stem cell identity and maintenance, whereas low levels enhance cell division and differentiation (Galinha et al. 2007). Two NAC transcription factors, Os06g0530400 (*OsNAC7*) and Os02g0745300, share sequence similarities with *SOMBRERO* (*SMB*) and *FEZ*, which are involved in root cap development in *Arabidopsis* (Willemsen et al. 2008). Both Os06g0530400 and Os02g0745300 showed a root cap-like pattern in the basal region of the embryo (Fig. 14.3a), suggesting that both genes may be involved in root cap development in radicle formation.

We also examined the expression patterns of Group I transcription factor genes in crown root primordia to check whether these patterns are similar to those in the radicle. Almost all of the genes examined had similar expression patterns, suggesting that they have the similar function during radicle and crown root formation (Fig. 14.4a). We also performed double-target in situ hybridization in crown root primordia, where the identities of cell layers are more easily distinguishable than in the radicle. First, we used the probe for *OsSCR1* and *AP2/ERF*s. *OsSCR1* mRNA was localized in the endodermis, including epidermis-endodermis initial cells, and in central QC cells (Fig. 14.4b). Os06g0657500 was expressed in the inner and outer layers of the endodermis without overlapping with the *OsSCR1* signal (Fig. 14.4b). Os02g0614300 was also expressed in the inner and

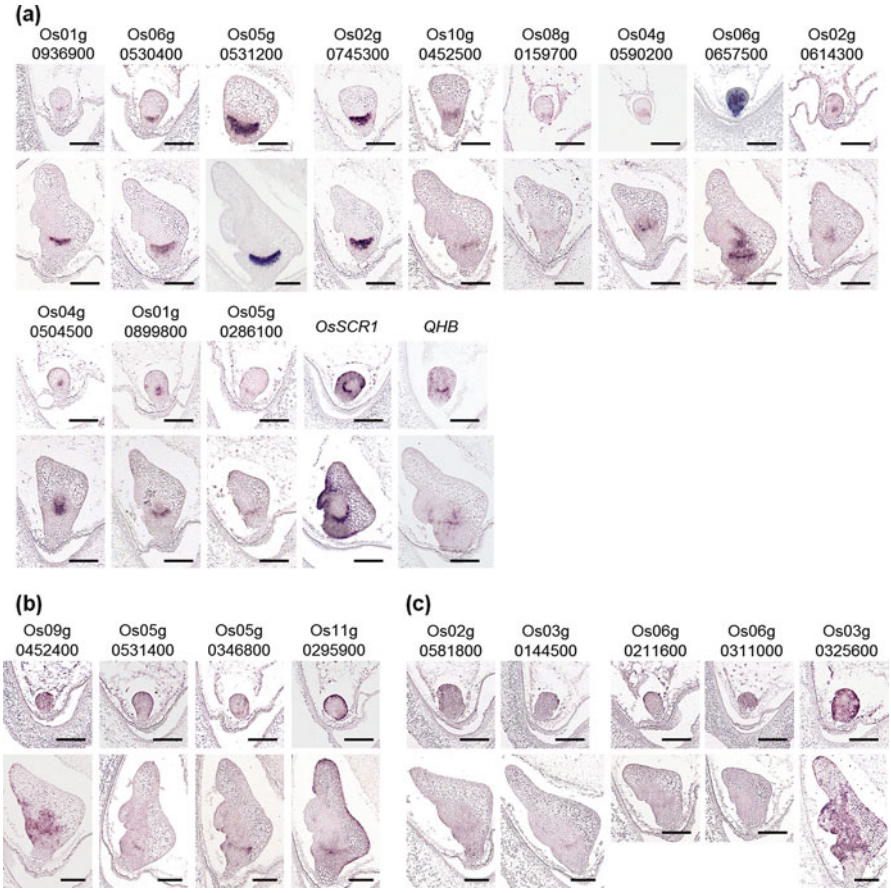

Fig. 14.3 Expression patterns of candidate genes essential for radicle formation in rice. (**a**) Group I: genes showing radicle-specific expression from radicle initiation to development. (**b**) Group II: genes showing radicle-specific expression during radicle development. (**c**) Group III: genes not showing radicle-specific expression. For each gene, a longitudinal section through a 3-DAP (days after pollination) to early 4-DAP embryo is shown in the top row and that through a late 4-DAP to 5-DAP embryo is shown in the *bottom row*. Signals were detected with DIG-NBT/BCIP. Bars = 100 μm. Of the 23 genes shown, 16 were selected as follows: 1st step, expression in the basal part of a 3-DAP embryo is >10 times that in the apical part according to microarray datasets in Itoh et al. (2016); 2nd step, high expression in the root of a 7-DAP embryo in Itoh's datasets; 3rd step, high expression in the root in a mature plant and embryo in a seed according to RiceXPro (Sato et al. 2011). Five transcription factor genes that are coexpressed with these 16 genes and are highly expressed in the radicle of a 7-DAP embryo were also chosen. Additionally, two genes (*OsSCR1* and *QHB*) previously reported to be expressed in the endodermis and central cells of QC were analyzed (Kamiya et al. 2003a, b)

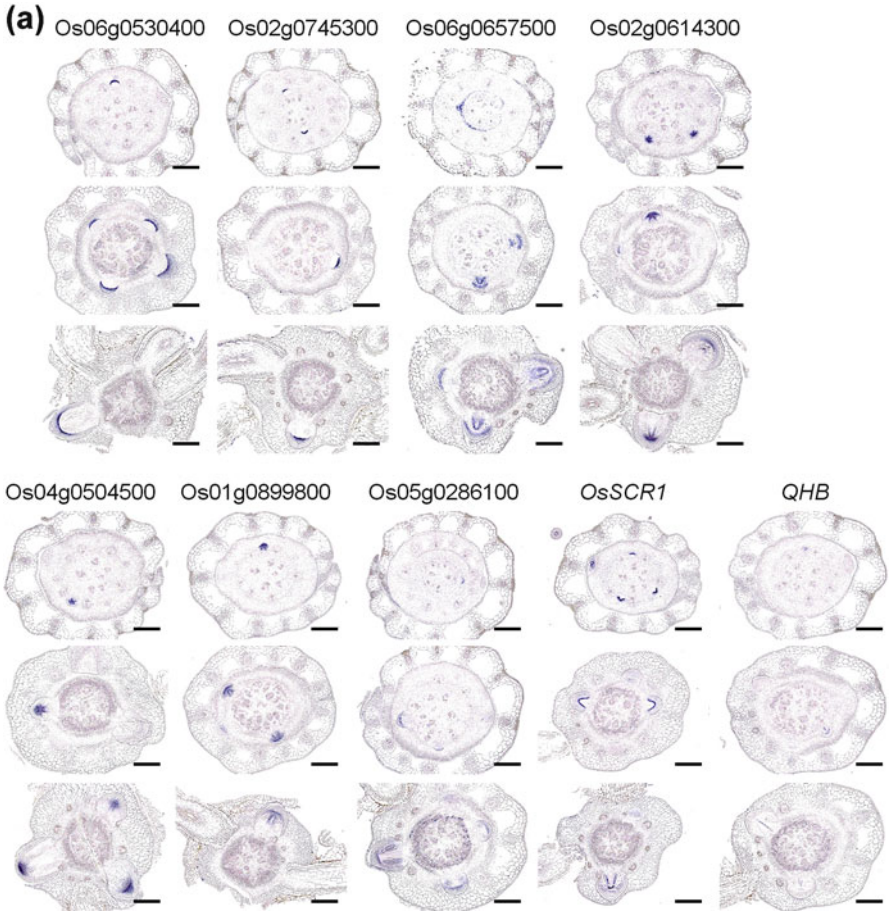

Fig. 14.4 Expression patterns of transcription factor genes in crown root primordia of rice. (**a**) Cross sections through the nodes of 7-day-old plants. Upper sections, initiation to early developing stage of crown root primordia. Middle sections, developing stage of crown root primordia. Lower sections, late developing to emergence stage. Signals were detected using DIG-NBT/BCIP. Bars = 200 μm. (**b**) Double-target in situ hybridization with probes for *OsSCR1* and *AP2/ERF* transcription factors. The *OsSCR1* signal was detected with biotin-TSA-fluorescein (green fluorescence), and Os06g0657500 and Os02g0614300 signals were detected with DIG-Fast Red (red fluorescence). Arrowheads indicate the central cells of QC. (**c**) Double-target in situ hybridization with the probes for NAC transcription factors. The Os02g0745300 signal was detected with biotin-TSA-fluorescein, and the Os06g0530400 signal was detected with DIG-Fast Red

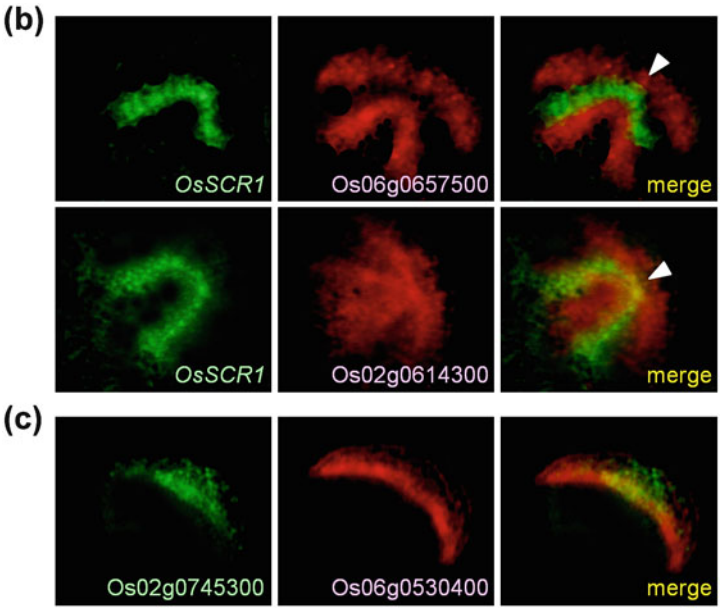

Fig. 14.4 (continued)

outer layers of the endodermis but overlapped with the *OsSCR1* signal in the stem cell region (Fig. 14.4b). The expression patterns of two *NAC*s were slightly different: the Os06g0530400 signal was observed in peripheral root cap cells and columella cells, whereas Os02g0745300 was expressed mainly in columella cells (Fig. 14.4c). Slight differences in the expression regions among the same gene family members might suggest the specialized function of each gene.

14.2.3 Crown Roots

Monocot plants produce numerous crown roots from nodes, which form a fibrous root system. During crown root morphogenesis, three developmental stages can be clearly distinguished: initiation, development, and growth (Coudert et al. 2010; Itoh et al. 2005; Kitomi et al. 2011; Zhao et al. 2009). The regulation of crown root formation in rice and that of lateral root formation in *Arabidopsis* share several common characteristics. The molecular mechanism of crown root formation is relatively well analyzed compared with that of other root types (Fig. 14.5).

Auxin is essential throughout root morphogenesis in these species, and auxin-related mutations lead to morphological abnormalities in rice crown roots and *Arabidopsis* lateral roots. Rice *YUCCA 1* (*OsYUC1*) and *CONSTITUTIVELY WILTED 1* (*OsCOW1*) encode flavin monooxygenases, the key enzymes in auxin

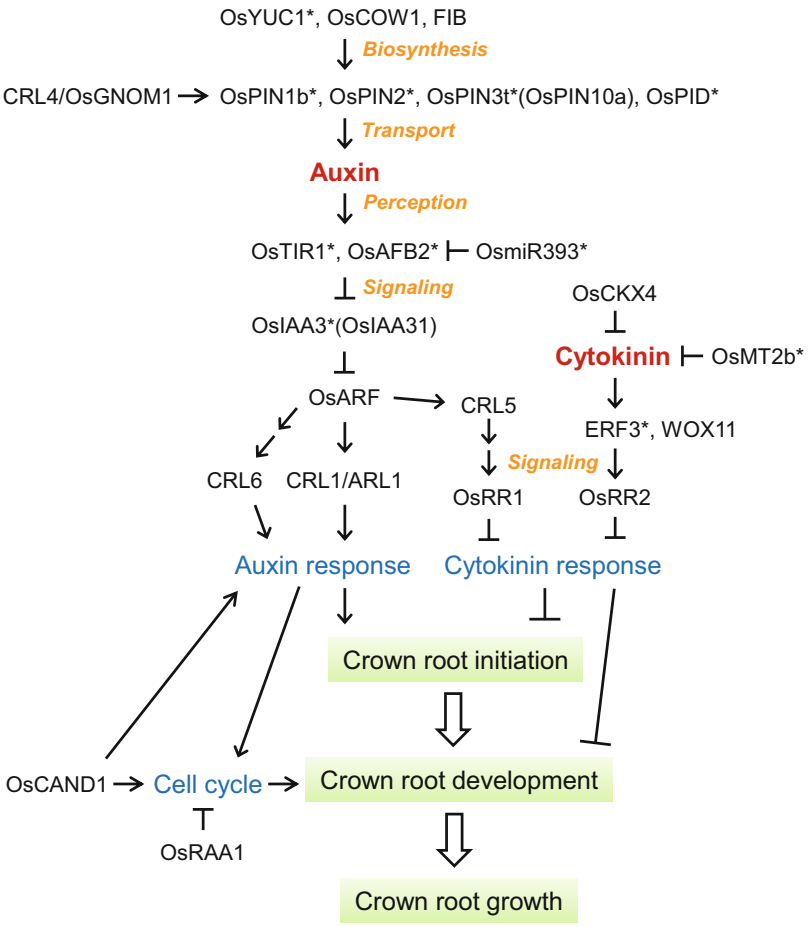

Fig. 14.5 Proposed gene regulatory pathways of crown root formation in rice. *Genes characterized by using reverse genetic approaches (overexpression, knockdown, or both). Genes without asterisks are cloned and characterized by using forward genetic approaches

biosynthesis (Woo et al. 2007; Yamamoto et al. 2007). Overexpression of *OsYUC1* enhances crown root formation, whereas *OsYUC1* antisense plants show severe growth retardation (Yamamoto et al. 2007). Alleles of *OsCOW1* with the insertion of the *Tos17* transposon or T-DNA decrease the root-to-shoot ratio by reducing crown and lateral root numbers (Woo et al. 2007). *OsCOW1* was also reported as *NARROW LEAF 7* (*NAL7*)/*OsYUC8*, which was identified in a mutant with narrow leaves (Fujino et al. 2008). The *fish bone* (*fib*) mutant defective in crown and lateral root formation has a mutation in the gene encoding TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (OsTAA1), an auxin biosynthetic enzyme (Yoshikawa et al. 2014). Several *PIN-FORMED* (*PIN*) genes, which encode auxin

efflux carriers mediating polar auxin transport, also play a pivotal role in crown root formation. Downregulation of *OsPIN1b* and *OsPIN3t* (termed *OsPIN10a* in Wang et al. 2009; *OsPIN3a* in Miyashita et al. 2010), and overexpression of *OsPIN2*, reduces crown root number (Chen et al. 2012b; Xu et al. 2005; Zhang et al. 2012a). Overexpression of *OsPINOID* (*OsPID*), which controls auxin distribution by controlling subcellular localization of PINs, also causes abnormal crown root development (Morita and Kyozuka 2007). Crown root initiation is impaired in *crown rootless4* (*crl4*)/*osgnom1* mutant (Kitomi et al. 2008b; Liu et al. 2009). GNOM is a membrane-associated guanine-nucleotide exchange factor for the G protein ADP-ribosylation factor (Arf-GEF) and plays an important role in polar auxin transport by establishing coordinated polar localization of PIN1 in *Arabidopsis* (Geldner et al. 2003; Steinmann et al. 1999). Distortion of polar auxin transport and altered expression patterns of *OsPIN*s were observed in *crl4*/*osgnom1* mutants, indicating that polar auxin transport is required for crown root initiation in rice.

Besides auxin biosynthesis and polar transport, auxin perception and signal transduction are essential for crown root formation. A rice microRNA, *miR393*, affects crown root initiation and seminal root development through negative regulation of the homologs of *Arabidopsis* auxin receptors *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*) and *AUXIN SIGNALING F-BOX 2* (*AFB2*), *OsTIR1*, and *OsAFB2* (Bian et al. 2012). Auxin signal is transmitted by a pathway mediated by Aux/IAA and AUXIN RESPONSE FACTOR (ARF) (Liscum and Reed 2002). Transgenic plants that produced constitutively active Aux/IAA, which was obtained by mutagenizing a conserved amino acid residue in the degradation-related domain (domain II) of OsIAA3 (OsIAA31 in Jain et al. 2006), have reduced crown root number (Nakamura et al. 2006). The *crl1*/*adventitious rootless1* (*arl1*) mutants develop few crown roots; *CRL1*/*ARL1* encodes a plant-specific LATERAL ORGAN BOUNDARIES DOMAIN (LBD)/ASYMMETRIC LEAVES2-LIKE (ASL) transcription factor, which acts downstream of the Aux/IAA and ARF-mediated auxin signaling pathway and whose expression is directly regulated by OsARF (Inukai et al. 2005; Liu et al. 2005). *CRL6*, which encodes a chromodomain helicase DNA-binding (CHD) family protein, is thought to influence crown root initiation and development through the Aux/IAA and ARF-mediated auxin signaling pathway because most of the 31 Aux/IAA genes are downregulated in the *crl6* mutant (Wang et al. 2016). The phenotype of the *crl2* mutant, impaired root gravitropism and crown root initiation, suggests that *CRL2* might also be involved in auxin signaling, although the causal gene has not yet been identified (Inukai et al. 2001; Yamamoto et al. 2010). Most of the auxin-related crown root mutants mentioned above also show defects in lateral root formation and root hair development, indicating the importance of auxin in overall root morphogenesis.

Cytokinin also plays an important role in the regulation of root morphogenesis and is widely known to act antagonistically to auxin: root formation is promoted by auxin but is suppressed by cytokinin. In *Arabidopsis*, root meristem size is controlled by the balance between cell differentiation and division, which results from antagonistic regulation by auxin and cytokinin (Dello Ioio et al. 2007, 2008). This

antagonistic regulation is also important in rice crown root formation. The phenotype of the dominant mutant *root enhancer1* (*ren1-D*), which has an increased crown root number, is caused by the activation of a *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) family gene, *OsCKX4* (Gao et al. 2014). CKXs are the only enzymes known to catalyze the irreversible degradation of cytokinin (Werner et al. 2003). Rice *METALLOTHIONEIN 2b* (*OsMT2b*) also has a role in the development of crown and lateral roots by influencing the endogenous cytokinin level (Yuan et al. 2008). Not only cytokinin content but also cytokinin signaling affects crown root formation. *CRL5* encodes an AP2/ERF transcription factor AINTEGUMENTA (ANT), and its expression is induced by OsARFs (Kitomi et al. 2011). Auxin-induced *CRL5* upregulates a type-A response regulator gene *OsRR1*, which suppresses cytokinin signaling and thus promotes crown root initiation. *WOX11* activates crown root development by directly repressing *OsRR2* (Zhao et al. 2009). Further analysis demonstrated that ERF3 interacts with WOX11 and promotes WOX11 binding to *OsRR2* (Zhao et al. 2015a).

Cell division is essential for crown root formation because it contributes to the development of crown root primordia. The *crl3* mutant produces a few crown root primordia consisting of vacuolated cells, whereas those in wild type consist of non-vacuolated cells (Kitomi et al. 2008a). Vacuolated cells divide in the early stage of crown root primordia development; however, cell division activity is gradually arrested, and primordia development is stopped in *crl3*. Overexpression of *O. sativa ROOT ARCHITECTURE ASSOCIATED 1* (*OsRAA1*) increases the number of crown and lateral roots compared with control plants (Ge et al. 2004). OsRAA1 is an anaphase-promoting complex/cyclosome (APC/C)-targeted protein to block the cell cycle at the transition from metaphase to anaphase (Han et al. 2008). A mutation in rice *CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED 1* (*OsCAND1*) causes a defect in the emergence of crown root primordia, although crown root initiation occurs normally (Wang et al. 2011). CAND1 is an SCF^{TIR1} E3 ubiquitin ligase involved in the degradation of Aux/IAA proteins in response to auxin in *Arabidopsis* (Chuang et al. 2004; Feng et al. 2004). *OsCAND1* is involved in auxin signaling to maintain the G2/M cell cycle transition in the crown root meristem and consequently the emergence of crown roots (Wang et al. 2011).

14.2.4 Lateral Roots

Molecular mechanisms of lateral root formation are similar to that of crown root formation; therefore, most of the crown rootless mutants show lateral rootless phenotype as well. However, the differences between crown root and lateral root obviously exist: the sites of their initiation, the number of inner cell layers, physiological functions, and plasticity in response to environmental stimuli (Luquet et al. 2005; Rebouillat et al. 2009; Suralta et al. 2008). Although most of the mutants lacking crown roots also lack lateral roots, some mutants have

abnormalities in lateral root formation without crown root defects. Analysis of such mutants might disclose lateral root-specific factors and schemes.

As mentioned above, auxin is a major player in lateral root formation. T-DNA-insertion mutants of the rice gene *AUXIN RESISTANT 1* (*OsAUX1*), which is evolutionarily close to the members of the auxin influx carrier gene family *AUX1/LIKE AUX 1* (*LAX*), have reduced lateral root number (Zhao et al. 2015b). The double mutant of *nal2* and *nal3* (*nal2/3*), which has mutations in two identical *OsWOX3A*/*OsNARROW SHEATH* (*OsNS*) genes located on chromosomes 11 and 12, respectively, produces fewer lateral roots than does the wild type (Cho et al. 2013). Reduced lateral root initiation in *nal2/3* seems to be attributable to compromised distribution of endogenous IAA caused by altered expression of *OsPIN1* and *OsPIN2*. Phenotypes of these mutants demonstrate that polar auxin transport mediated by auxin influx and efflux carrier proteins is important for lateral root formation. The phenotypes of some mutants also indicate the importance of auxin signaling mediated by Aux/IAA and ARF in lateral root formation. The gain-of-function mutants *osiaa11* and *osiaa13*, which have stabilizing mutations in domain II of Aux/IAA proteins, have dramatically reduced lateral root number (Kitomi et al. 2012; Zhu et al. 2012). The rice *cyclophilin 2* (*oscyp2*) mutant also shows impaired lateral root initiation (Kang et al. 2013). *OsCYP2* is involved in Aux/IAA degradation by stimulating the activity of the SCF^{TIR} ubiquitin E3 ligase complex. Auxin signaling is likely disturbed in these mutants because degradation of Aux/IAA proteins allows auxin-responsive transcription to be regulated by ARF proteins, which then act as transcriptional activators or repressors (Gray et al. 2001). A mutation in rice *HEME OXYGENASE* (*OsHO1*), which encodes an enzyme that catalyzes the degradation of heme into biliverdin IXa, Fe^{2+}, and carbon monoxide, also affects lateral root formation in a manner dependent on auxin and stress-related signals (Chen et al. 2012a). Some mutants with auxin-related abnormalities also have defects in lateral root formation; these include *lateral rootless 1* (*lrt1*), *lrt2*, *auxin-resistant mutant 1* (*arm1*), and *arm2* (Chhun et al. 2003; Faiyue et al. 2010; Hao and Ichii 1999; Wang et al. 2006b).

Cell cycle regulation is necessary for lateral root development. The mutant of *O. sativa ORIGIN RECOGNITION COMPLEX SUBUNIT 3* (*OsORC3*) has a temperature-dependent defect in lateral root development (Chen et al. 2013). In *OsORC3* knockdown plants, the emergence of lateral root primordia is blocked due to the perturbation of cell cycle-related gene expression in the primordia (Chen et al. 2013).

14.2.5 Root Hairs

Root hairs are long cylindrical outgrowths of individual root epidermal cells and are thus different from seminal, crown, and lateral roots. Root hair development includes three stages: cell fate determination, root hair initiation, and root hair elongation (Huang et al. 2013b). Vesicle trafficking, cytoskeleton reorganization,

and cell wall loosening and synthesis are major driving forces for root hair elongation that depend on gene expression promoted by signals such as auxin, cellular pH, calcium ions, extracellular reactive oxygen species (ROS), and phosphatidylinositols (Libault et al. 2010).

A mutation in *O. sativa SEC14-NODULIN DOMAIN PROTEIN* (*OsSNDP1*), which encodes a phosphatidylinositol transfer protein, leads to short-branched root hairs (Huang et al. 2013b). Similar defects were reported in *Arabidopsis* mutants with defects in phospholipid metabolism and signaling (Kusano et al. 2008; Vincent et al. 2005), indicating the critical roles of phospholipids in root hair elongation. *ROOT HAIRLESS 1* (*RTH1*)/*O. sativa APYRASE 1* (*OsAPY1*), which encodes an enzyme that hydrolyzes NTPs and/or diphosphates, also affects root hair elongation (Yuo et al. 2009). Apyrases control the concentration of extracellular ATP, which functions as a signal molecule for growth control and is localized in the regions of active growth and cell expansion such as root hair tips (Roux and Steinebrunner 2007; Wu et al. 2007). Rice *FORMIN HOMOLOGY 1* (*OsFH1*) is important for root hair elongation under submerged conditions (Huang et al. 2013a). Formins play critical roles in cytoskeleton organization by nucleating actin polymerization and elongation and bundling actin filaments, which drive tip growth (Paul and Pollard 2009). *OsFH1* is assumed to have similar functions, although no null mutant with a defective root hair phenotype has been reported in *Arabidopsis* or rice (Deeks et al. 2005; Yi et al. 2005).

Factors involved in cell wall modification are closely related to root hair development. Root hair length is reduced in the rice *short root hair 2* (*srh2*) mutant, which has a mutation in the *XYLOGLUCAN XYLOSYLTRANSFERASE 1* (*OsXXT1*) gene (Wang et al. 2014a). Xyloglucan is not considered to be an important component of cell wall in grasses, including rice, because its content is below 5% (Vogel 2008). However, the *srh2* mutant demonstrates the importance of xyloglucan in rice root hair development. Expansins, which are associated with cell wall loosening, permit turgor-driven cell elongation (Cosgrove 2000). Rice *EXPANSIN A17* (*OsEXPA17*) and *OsEXPA30* are also involved in root hair elongation because the *osexpA17* mutant shows defects in root hair elongation, and these defects are partially complemented by *OsEXPA30* (Yu et al. 2011). The promoters of *OsEXPA17* and *OsEXPA30* contain conserved root hair-specific *cis*-elements (RHEs), which are also found in root hair-specific genes and genes paralogous to *AtEXPA7* (Kim et al. 2006). These *EXPA*s with RHEs are expressed in a root hair-specific manner in *Arabidopsis* and rice (Kim et al. 2007; Yu et al. 2011). *OsEXPB5*, which is so far found in Gramineae family and is absent in dicots, also has RHEs in the promoter region, and its expression is strongly associated with root hair initiation and elongation (Won et al. 2010); however, its in vivo function in root hair development has not been demonstrated. *RTH2*/*O. sativa CELLULOSE SYNTHASE-LIKE D1* (*OsCSLD1*) is also required for root hair elongation (Kim et al. 2007; Yuo et al. 2011). Only *OsCSLD1* is specifically expressed in roots, similar to root hair-specific genes with RHEs, whereas other *OsCSLD* subfamily members are expressed in both roots and shoots.

The root epidermis comprises hair cells (trichoblasts) and non-hair cells (atrichoblasts). In each plant species, root hair patterning belongs to one of three types according to the way how the fate of each cell is determined (Kim et al. 2006). In Type 1, hair cells can differentiate from any epidermal cell. In Type 2, the root epidermis consists of cells of two sizes, and only the short cells differentiate into hair cells. In Type 3, hair cells produce rows along the longitudinal root axis, resulting in a striped pattern. Root hair patterning in rice is Type 2, and the differences in size between mature hair and non-hair cells result from differential cell expansion relatively late in the development, after initiation of root hair growth (Kawata and Ishihara 1959; Kim and Dolan 2011). A mutation in the *O. sativa ROOT HAIRLESS 1* (*OsRHL1*) gene, which encodes a basic helix-loop-helix (bHLH) transcription factor, results in very short root hairs (Ding et al. 2009). In the *osrhl1* mutant, clearly short and long epidermal cells characteristic of Type 2 species are not observed, suggesting that *OsRHL1* controls root hair elongation and epidermal cell patterning, similar to the bHLH gene *ROOT HAIR DEFECTIVE 6-LIKE 4* (*RSL4*) in *Arabidopsis* (Ding et al. 2009; Yi et al. 2010).

14.3 Formation of Root System Architecture

The outline of the rice root system is formed by multiple crown roots developed from several phytomers; a phytomer is a nodal unit consisting of a leaf, an axillary bud, and crown roots (Rebouillat et al. 2009). Crown roots developed from the upper and lower regions of each node are called the upper and lower crown roots, respectively (Fig. 14.2). The lower crown roots have a larger diameter than the upper crown roots (Abe and Morita 1994). Another feature of lower crown roots is downward elongation, whereas upper crown roots elongate randomly in directions ranging from lateral to vertical, suggesting that they respond to gravity more weakly than do the lower crown roots (Abe and Morita 1994). Overall, the growth angle of each upper and lower crown root determines vertical distribution of the whole root system in the soil. Shallow and steep root growth angles favor root distribution in the topsoil and subsoil, respectively. The maximum length of each crown root restricts range of access for absorption of water and nutrients from the soil. Short roots result in compact root systems, whereas long roots produce large root systems.

The genetic mechanism of root system development in rice has been dissected mainly on the basis of QTL analysis; the first such study was reported by Champoux et al. (1995). Hundreds of QTLs with small to intermediate genetic effects on many root parameters that affect root system architecture have been detected in rice; such parameters include the growth angle, length, volume, and thickness of the roots (Rebouillat et al. 2009). However, the genetic mechanisms underlying these QTLs are poorly understood. On the other hand, several genes for root development have been isolated in rice mutants showing abnormal root phenotypes (Rebouillat et al.

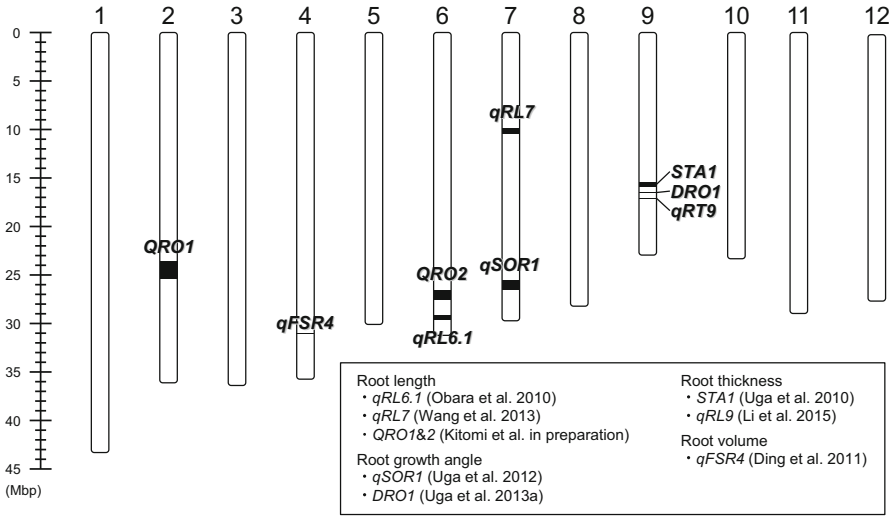

Fig. 14.6 Location of rice QTLs involved in root system architecture that are potentially useful for molecular breeding. Chromosome numbers are indicated above each linkage map. Black box indicates a region that contains a root QTL

2009; Wu and Cheng 2014). In this section, we discuss the genes and QTLs related to quantitative variation of root system architecture in rice (Table 14.1; Fig. 14.6).

14.3.1 Root Growth Angle

Root growth angle is controlled by several environmental factors such as gravity, light, and water potential (Oyanagi et al. 1993; Uga et al. 2015a). Root gravitropism has been well studied in *Arabidopsis* (Baldwin et al. 2013; Morita 2010), but not in monocot plants including rice. Only two QTLs for the root gravitropic response have been reported in rice (Norton and Price 2009), but the underlying genes have not yet been isolated. *DRO1*, which was reported originally as a major QTL responsible for root growth angle, is also involved in gravitropism (Uga et al. 2013a). *DRO1* is negatively regulated by auxin signaling downstream of Aux/IAA and ARF and is involved in cell elongation in the root tip, which causes gravitropic bending (Uga et al. 2013a). Under normal growth conditions, *DRO1* is expressed around the RAM in the root tip and crown root primordia. For the response to gravitropic stimuli (i.e., rotation of the roots from the normal vertical axis to the horizontal axis), *DRO1* transcripts in the outer cells of the distal elongation zone are repressed on the lower side than on the upper side of the roots by the redirected auxin flow to the lower side of the root, resulting in decreased cell elongation in the lower side relative to the upper side. This process contributes to asymmetric

growth, leading to root gravitropic bending. Thus, QTLs for gravitropism should affect root growth angle, resulting in a natural variation of root system architecture.

Genes with high sequence similarity to *DRO1* have been found in other monocots such as maize, sorghum, and barley, but their physiological and molecular functions are still unknown (Uga et al. 2013a). Recently, genes with low sequence similarity to DRO1 have been identified in dicots. The legume *Medicago truncatula* carrying mutations in *NEGATIVE GRAVITROPIC RESPONSE OF ROOTS* (*NGR*) shows a negative root gravitropic response (Ge and Chen 2016). Only triple mutants of three redundant *AtNGR* genes (At1g17400, At1g72490, At1g19115) in *Arabidopsis* also showed a similar negative root gravitropic response (Ge and Chen 2016). These *NGR* genes may be *DRO1* homologs in the IGT family, the members of which have relatively low sequence similarity to each other but have conserved amino acid motifs (Guseman et al. 2017). These findings suggest that the functions of *DRO1* and *DRO1* homologs in root gravitropism are conserved in monocots and dicots. The IGT family also includes *TILLER ANGLE CONTROL 1* (*TAC1*) and *LAZY1*, which control the branching angle of lateral shoot organs in both monocots and dicots (Guseman et al. 2017), suggesting that this gene family might be associated with the regulation of growth angle in shoot and root organs.

Many other QTLs for root growth angle have been reported in rice (Kitomi et al. 2015; Lou et al. 2015; Uga et al. 2012, 2013b, 2015b). *DRO2* (Uga et al. 2013b), *DRO3* (Uga et al. 2015b), *DRO4* (Kitomi et al. 2015), and *DRO5* (Kitomi et al. 2015) were detected in seven F_2 mapping populations derived from a cross between several rice accessions with different root growth angles and "Kinandang Patong" as a donor line with a large root growth angle. Therefore, QTLs associated with root growth angle distinct from *DRO1* exist in "Kinandang Patong." *qSOR1* (*quantitative trait locus for SOIL SURFACE ROOTING 1*) has been detected on chromosome 7 in recombinant inbred lines derived from a cross between "Gemdjah Beton," a lowland rice accession with a high proportion of crown roots that run along or near the soil surface, and "Sasanishiki," a lowland rice accession that does not form soil-surface roots (Uga et al. 2012). The "Gemdjah Beton" allele of *qSOR1* causes many thick crown roots to elongate near the soil surface from the seedling stage. This phenotype is very unique because thick crown roots generally elongate downward. *qSOR1* was fine-mapped to a 812-kb candidate region on chromosome 7 (Uga et al. 2012; Fig. 14.6). Lou et al. (2015) also reported QTLs for root growth angle on chromosomes 1, 2, 4, 7, and 10. Among them, three QTLs on chromosomes 2, 4, and 7 are located near the regions of *DRO4*, *DRO2*, and *qSOR1*, respectively. Cloning of these QTLs would deepen our understanding of the genetic mechanisms that determine root growth angle in rice.

14.3.2 Root Length

Maximal root length is determined by the rate and duration of root elongation. Root elongation is caused by cell division and elongation. Mutant analyses revealed that

genes related to cell wall growth, cell expansion, and auxin signaling are involved in the division and elongation of root cells. Cell wall development affects root elongation and the maintenance of root structure. *OsDGL1* encodes the dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48-kDa subunit precursor (Qin et al. 2013). An ethyl methanesulfonate (EMS)-induced *osdgl1* mutant has a defect in *N*-glycosylation, an altered composition of matrix polysaccharides in the cell wall, and cell death in the root, resulting in a decrease in root elongation without a decrease in the numbers of crown roots, lateral roots, or root hairs. *OsMOGS* encodes a putative mannosyl-oligosaccharide glucosidase and acts downstream of *OsDGL1* during *N*-glycan processing in the endoplasmic reticulum (Wang et al. 2014b). An EMS-induced *osmogs* mutant has a decreased cell division and elongation in the root, resulting in short roots. OsMOGS is needed for cellulose biosynthesis and OsABCB-mediated auxin transport in rice (Wang et al. 2014b). Other genes associated with cell wall modification also control root elongation. *ROOT GROWTH INHIBITING* (*RT*)*/OsGLU3*, which encodes a membrane-anchored endo-1,4-β-D-glucanase, is involved in cell wall loosening necessary for root cell elongation (Inukai et al. 2012; Zhang et al. 2012b). The *rt./osglu3* mutants have short-root phenotypes due to a decrease in longitudinal cell elongation without changes in root differentiation, root cell division, or shoot development. Interestingly, cellulose content in roots is increased in an ethylene imine-induced *rt*. mutant (Inukai et al. 2012) but is decreased in EMS-induced *osglu3* mutants (Zhang et al. 2012b). To reconcile these contradictory findings, further studies are needed. *OsGNA1* encodes glucosamine-6-P acetyltransferase, which is involved in de novo UDP-*N*-acetylglucosamine biosynthesis (Jiang et al. 2005). A T-DNA insertion *osgna1* mutant has decreased root cell elongation caused by cell shrinkage, perhaps because of insufficient UDP-GlcNAc for protein *N*-glycosylation, which is necessary for plant development including cell wall synthesis (Lerouge et al. 1998; Lukowitz et al. 2001). Cell expansion occurs in turgor-driven cell elongation. Transgenic plants overexpressing *OsEXPA8*, which encodes a root-specific α-expansin (Shin et al. 2005), have increased seminal, crown, and lateral root length as well as plant height and increased leaf number and size caused by an increase in cell length in both shoot and root vascular bundles (Ma et al. 2013).

Auxin regulates cell fate determination and cell elongation (Tanaka et al. 2006). These effects are mostly mediated by ARFs. Loss-of-function *Tos17* and T-DNA insertion mutants of *osarf12*, which is a member of ARFs (Wang et al. 2007), have short-root phenotypes due to a smaller elongation zone in seminal roots compared to the wild type (Qi et al. 2012). The short elongation zone is likely caused by a low auxin concentration. *O. sativa SHORT POSTEMBRYONIC ROOTS 1* (*OsSPR1*) encodes a putative mitochondrial protein with an Armadillo-like repeat domain (Jia et al. 2011). EMS-induced *osspr1* mutants have short-root phenotypes (decreased lengths of seminal, crown, and lateral roots) due to reduced cell elongation, whereas lateral root initiation and lateral root number are similar to those in the wild type. *OsCYT-INV1*/*OsNIN8* encodes alkaline/neutral invertase and is homologous to *AtCYT-INV1* in *Arabidopsis* (Ji et al. 2005, Jia et al. 2008). An EMS-induced *Oscyt-inv1* mutant has a short-root phenotype due to a decreased cell length

probably caused by hexose deficiency, as hexoses play various roles in cell elongation.

Despite isolation of several genes, our knowledge of the genetic mechanism controlling root length in rice is still limited compared to that for *Arabidopsis*. Reverse genetics should be a valuable approach to gain a better understanding of the gene network that regulates root length. Antisense transgenic plants with downregulated *OsCKI1*, which encodes putative casein kinase I, have short seminal roots and a low number of crown and lateral roots caused by reduced cell elongation (Liu et al. 2003). Examination of transgenic rice plants over- and under-expressing *OsRPK1*, which encodes an LRR-RLK, revealed that this gene affects seminal root length and crown root number by negatively regulating polar auxin transport (Zou et al. 2014).

Several QTLs for root length have been fine-mapped in rice, although none of them have been cloned (Fig. 14.6). *qRL6.1*, a QTL controlling root length at the seedling stage under hydroponic conditions, was mapped to a 337-kb interval on chromosome 6 (Obara et al. 2010). *qRL7*, a QTL affecting root length at the heading stage under hydroponic conditions, was mapped to a 657-kb interval on chromosome 7 (Wang et al. 2013). Recently, *QUICK ROOTING 1* (*QRO1*) and *QRO2* have been fine-mapped on chromosomes 2 and 6, respectively, in chromosome segment substitution lines derived from a cross between "IR64" and "Kinandang Patong" and grown under hydroponic conditions (Kitomi et al. in press, Fig. 14.6).

14.3.3 Other Root Traits

The combination of growth angle and length in seminal and crown roots is the main determinant of root system architecture in cereals (Abe and Morita 1994; Araki et al. 2002), although other root traits such as volume and thickness are also important.

Root volume affects root surface area and thus absorption of water and nutrients from soil (Gowda et al. 2011; Wang et al. 2006a), but rice genes that control root volume have not yet been isolated. *qFSR4*, a QTL for root volume per tiller, has been fine-mapped on chromosome 4 (Ding et al. 2011). The 38-kb *qFSR4* candidate region has three open reading frames including *NAL1* (Qi et al. 2008). The *NAL1* gene is associated with polar auxin transport and controls leaf width. *qFSR4* also affects flag leaf width. *NAL1* may be the most promising candidate gene for *qFSR4* because polar auxin transport affects root development and shoot growth.

Root thickness affects uptake of water and nutrients as well as root penetration ability (Gowda et al. 2011; Wang et al. 2006a). *qRT9*, a QTL for root thickness, has been fine-mapped to an 11.5-kb candidate region on chromosome 9 (Li et al. 2015) with only one annotated open reading frame, Os09g0455300, which encodes a

putative bHLH transcription factor (OsbHLH120). Haplotype and expression analyses suggest that *OsbHLH120* is the candidate gene for *qRT9*. For water and nutrient translocation, stele and xylem structures should be more important than root thickness (Uga et al. 2008). *STELE TRANSVERSAL AREA 1* (*STA1*), a QTL controlling stele transversal area, has been fine-mapped to a 359-kb interval between SSR markers RM566 (14.70 Mb) and RM24334 (15.06 Mb) on chromosome 9 (Uga et al. 2008, 2010). *qRT9* (17.13 Mb) and *STA1* are located near *DRO1* (16.31 Mb). As mentioned above, upland rice tends to have deeper and thicker roots than those of lowland rice (O'Toole and Bland 1987). The tight linkage of these QTLs should be associated with the phenotypic relationship between these root traits.

14.4 Conclusions

Several rice-specific genes controlling the root system have been found. However, the many rice genes homologous to *Arabidopsis* genes associated with the formation of the main root system are also involved in the formation of the fibrous root system. Thus, many parts of the genetic mechanism related to the root system have features common between monocots and dicots. On the other hand, much remains to be clarified about the difference between monocots and dicots in the natural variation of root system architecture because most related genes have not been cloned. Because dicots have *DRO1* homologs, their genetic mechanism related to the natural variation in root system architecture might have many common features with that of monocots. Recent progress of forward and reverse genetic strategies, such as MutMap (Abe et al. 2012; Takagi et al. 2015) and TILLING (Suzuki et al. 2008; Till et al. 2007), and the CRISPR/Cas9-mediated genome editing system (Doudna and Charpentier 2014; Schaeffer and Nakata 2015) allow us to isolate genes and find new alleles easily. Using QTL cloning and these approaches, we would be able to elucidate the entire genetic network related to natural variation in rice root system architecture. Understanding of the genetic mechanism of root plasticity in response to environmental variation is also important for improving crop production under abiotic stresses, but it is difficult to obtain reliable phenotypic data and identify related genes or QTLs under field conditions. To resolve this issue, a reproducible root phenotyping platform with controlled soil water, nutrients, and temperature in which we can evaluate accurately a large number of plants is needed.

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Chapter 15
Genetics and Breeding of Flooding Tolerance in Rice

Endang M. Septiningsih and David J. Mackill

Abstract Flooding is a frequent natural calamity, affecting global food supply and financial security. The intensity of rainfall events is expected to increase under future climate change scenarios, which will greatly impact rice production. Different flooding patterns can cause damage or complete yield loss in rice plants at different stages of growth. This includes (a) complete submergence due to flash flood at vegetative or pre-flowering stages, (b) stagnant flooding of medium-deep water and deepwater or floating rice, and (c) submergence at germination or anaerobic germination. Different molecular and physiological mechanisms underly tolerance to each type of flooding. Several major QTLs have been mapped and several key genes underlying the QTLs have been cloned. Remarkable progress has been achieved through conventional and molecular breeding strategies in developing tolerant varieties to mitigate the impact of different flood events. This effort will be continued in the future by incorporating new QTLs/genes and tolerance to other abiotic and biotic stresses according to the needs of the target regions. Genetics, genomics, and other modern technologies will also be continuously explored to further our understanding of how rice plants cope with different types of flooding stress.

Keywords Flooding tolerance · Sub1 varieties · Stagnant flooding · Deepwater rice · Anaerobic germination · QTLs · Genes · Molecular breeding

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15.1 Introduction

Rice cultivation and flooding are considered synonymous. The ancestors of modern rice cultivars were aquatic species and provided a suitable basis for development of agriculture in swampy monsoonal environments. The floods bring water and nutrients to the rice plants, but can also kill the plants if too deep or prolonged.

Flooding is the most destructive natural calamity. For example, in the first half of 2013, it was estimated that floods were responsible for 47% of the damage from all natural disasters (Wake 2013). The specter of climate change, with images of violent storms, has increased concern over damage caused by flooding. An escalation in "record-breaking" rainfall events in recent decades has been recorded (Lehmann et al. 2015). While the frequency of tropical cyclones has declined, their intensity has increased (Auffhammer et al. 2012; Kang and Elsner 2015). There is evidence that rice-growing areas will be especially impacted by high rainfall storms in the future (Cai et al. 2014; Hirabayashi et al. 2013; Singh et al. 2014). However, rice farmers have been concerned about survival of their crop under flooding long before the modern worries of climate change. Likewise, rice scientists have been studying how rice plants can cope with excess flooding over many decades.

The different flooding patterns affecting rice plants discussed in this review article are shown in Fig. 15.1. Three major types of flooding require different coping mechanisms in rice plants: (1) short-duration flooding of less than 3 weeks, referred to as submergence; (2) long-duration flooding, sometimes referred to as stagnant flooding; and (3) flooding during germination, referred to as anaerobic germination.

With shorter-term flooding, the plants are submerged for up to a few weeks (Fig. 15.1d). Perhaps the first mention of submergence tolerance in the scientific era is by Graham (1913), who classified Indian flood-tolerant varieties as those that could survive up to 15 days under water. Scientists in India knew of these varieties and selected two famous pure-line selections FR13A and FR43B from the farmers' traditional landraces *Dhalputtia* and *Bhetnasia*, respectively (Richharia and Govindaswami 1966). We do not have the exact year of their selection, but these probably were isolated at the Cuttack station, Odisha, before this was turned over to the Central Government in 1946. The Indian flood-tolerant varieties such as FR13A and FR43B are botanically *aus* varieties, although they differ from typical *aus* varieties in being photoperiod sensitive.

With stagnant flooding (Fig. 15.1b, c, e), the water depth can vary greatly. Deepwater rice (flooding deeper than 50 cm for at least 1 month in the season) is an ancient type of rice culture that originated in both Asia, from the *Oryza sativa* species, and Africa, from the *O. glaberrima* species (Catling 1992). The rice varieties grown in these areas need to elongate rapidly during the onset of the floods to keep part of the plant above the water level. Large areas of rice production are subject to medium-deep (Fig. 15.1b, e), stagnant flooded conditions, where water depths of 25–50 cm have adverse effects on rice yields (Singh et al. 2011).

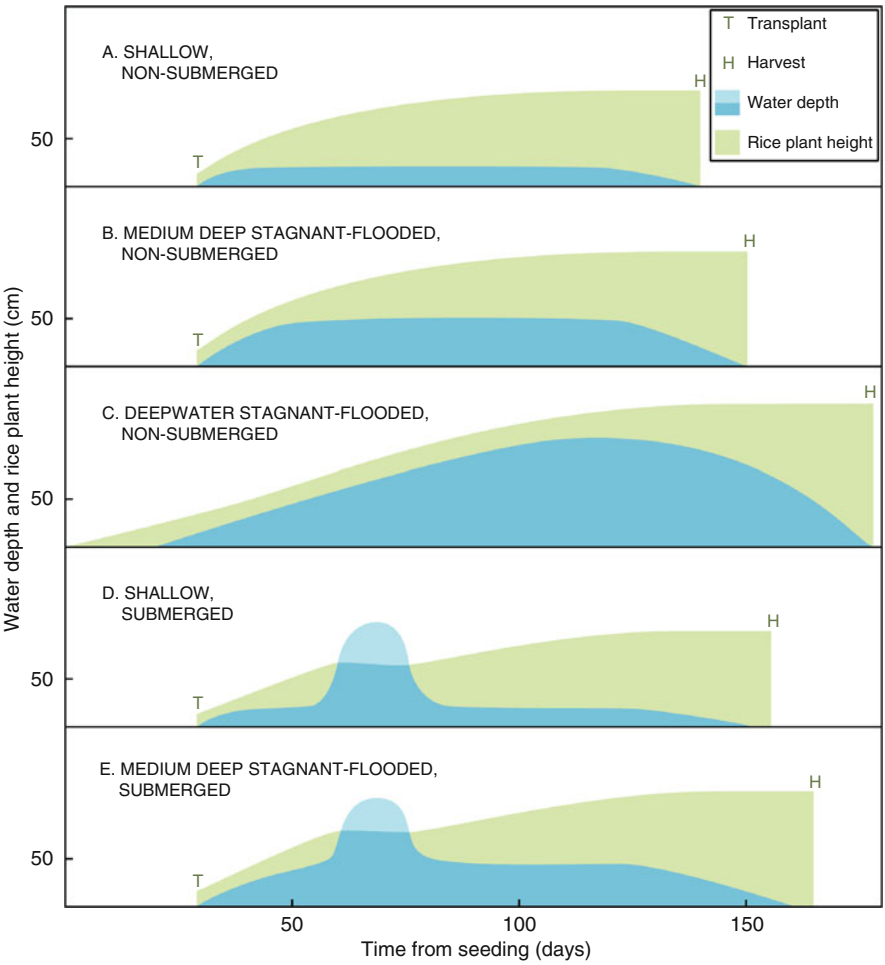

Fig. 15.1 Flooding patterns in rice. In shallow, irrigated rice (**a**), water depth usually remains well below 25 cm for the crop duration. In medium-deep conditions (**b**), water depths can remain between 25 and 50 cm for prolonged periods, and in deepwater and floating rice (**c**), water depths can go to 1 m or more for an appreciable period of the season and the rice is direct-seeded in the field. Submergence is a short-term stress of 2–3 weeks and can occur in shallow flooded (**d**) or stagnant flooded (**e**) conditions

For the third type of flooding, in areas where rice is seeded directly into the field, submergence can occur during the germination stage (Ismail et al. 2009; Yamauchi et al. 1994). This is becoming increasingly relevant as more rice-producing areas shift to direct-seeded systems.

After the formation of the International Rice Research Institute (IRRI) in the Philippines in 1960, scientists began organizing meetings to focus research on flooding stresses. International deepwater rice symposia were held in 1974

(Dhaka), 1976 (Bangkok), 1981 (Bang Khen), and 1987 (Bangkok), and their proceedings are available at https://books.google.com/. It was also recognized that flash flood causing submergence stress was a major problem in some irrigated and rainfed lowland areas, and this was also addressed in an international meeting held at Bhubaneswar, India, in 1985 (IRRI 1986). The International Rice Testing Program (later renamed the International Network for Genetic Enhancement of Rice or INGER) created nurseries addressing submergence-prone and deepwater rice, and international collaboration in breeding increased, resulting in the development of many new varieties (Mackill et al. 2013).

15.2 Genetic Networks Controlling Tolerance to Flooding Stresses

To consider the genetics and breeding of tolerance to flooding, it is critical to recognize how rice plants can survive and produce optimum yields under the different types of flooding stresses:

- Submergence at vegetative or pre-flowering stage (submergence)
- Stagnant flooding of medium-deep (25–50 cm), deepwater (50–100 cm), or floating rice (>100 cm)
- Submergence at germination (anaerobic germination, AG)

Submergence is mainly a problem in shallow to medium-deep lowland rice, but it can also occur in deepwater areas. Likewise, stagnant flooding often occurs in areas of medium-deep and deepwater systems, where poor drainage in the fields combined with high rainfall prevents the water levels from receding for weeks, or even months, at a time. Anaerobic germination is mainly required where rice is seeded directly into fields, although for transplanted rice submergence at germination can occur also in the seedbeds. Very few rice varieties can germinate when fields are flooded after seeding. There has also been interest in developing this trait for intentionally flooding fields after sowing as a means of weed control (Williams et al. 1990).

Each of these stresses can be addressed independently, and the genes conferring tolerance can be combined to confer multiple tolerances. Adaptation to stagnant flooding is not as well understood. However, for areas with deeper flood levels, rapid internode elongation is necessary, and this trait is not compatible with submergence tolerance.

15.2.1 *Submergence Tolerance at the Vegetative and Early Reproductive Stage:* SUB1 *and Other Genes*

Submergence tolerance in the highly tolerant FR13A (and its progeny) was found to be controlled by the *SUB1* QTL on chromosome 9 (Xu and Mackill 1996). The locus contains three ethylene responsive factors (ERFs), *SUB1A*, *SUB1B*, and *SUB1C* (Xu et al. 2006). All rice varieties studied have the *SUB1B* and *SUB1C* genes, while *SUB1A* is present in a subset of *indica* or *aus* rice varieties (Xu et al. 2006). Subsequently, it has been confirmed that within the *SUB1* gene cluster, *SUB1A* was the key determinant for complete submergence tolerance (Septiningsih et al. 2009; Xu et al. 2006). Submergence injury at the vegetative stage is increased by rapid growth, which depletes carbohydrate reserves. *SUB1A* uses a "quiescence" strategy that restrains elongation and conserves carbohydrate reserves (Fig. 15.2) (Fukao et al. 2006). The plant is consequently able to rapidly recover and regrow new tillers when the water recedes.

The frequency of the tolerant allele of *SUB1A* in rice germplasm is surprisingly low, considering the low number of submergence donors identified from numerous germplasm screenings performed at IRRI—probably less than 1%. However, based on our findings so far, this allele does not have any yield penalty or other negative effects on other important agronomic traits, and it is hypothesized that this locus was not selected against by farmers. Two common sources of tolerant *SUB1A* genes are the *aus* rices from Odisha, including FR13A and FR43B, and submergence-tolerant indica rices from Sri Lanka, including Kurkaruppan, Goda Heenati, and Thavalu (Vergara and Mazaredo 1975), and at least some of these seem to share the common tolerant haplotype for the *SUB1* locus (Singh et al. 2010). Most likely the tolerance gene originated in the *aus* rice of Odisha and was brought to Sri Lanka through migration, ultimately ending up in the *indica* landraces. Odisha has been mentioned as a possible origin for the Sinhalese people of Sri Lanka in the fifth century BC (Roychowdhury 2016; Senaveratna 1997). The *SUB1A* gene has been found in wild rice progenitor *Oryza rufipogon* (Li et al. 2011). More recently, dos Santos et al. (2017) reported that the *SUB1* locus is present in most *Oryza* species and that the *SUB1A* gene of *O. nivara* is on chromosome 1.

Varieties with and without the *SUB1A* tolerance gene generally show contrasting levels of tolerance (see Fig. 15.4), but there are differences in the degree of submergence tolerance in varieties with the *SUB1* gene that are under the control of other genes. A few smaller QTLs for submergence tolerance during vegetative stage have been identified (Gonzaga et al. 2016, 2017; Nandi et al. 1997; Septiningsih et al. 2012; Toojinda et al. 2003). It is possible that the tolerance mechanisms underlying some of these QTLs are different from *SUB1;* further investigation is needed to prove this hypothesis.

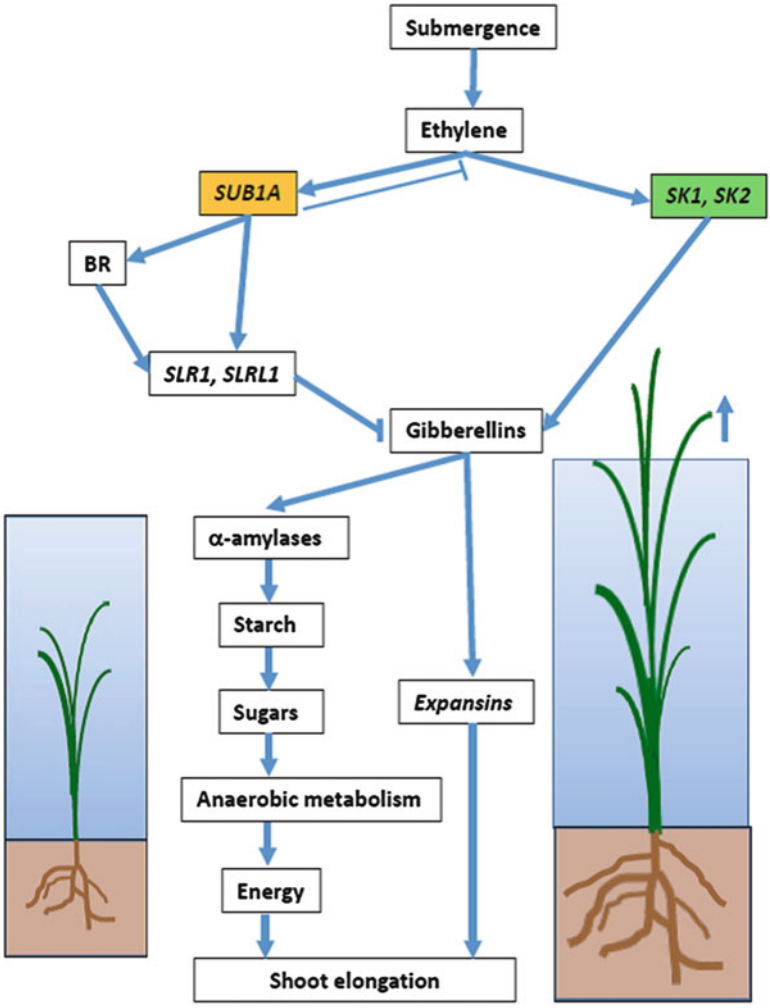

Fig. 15.2 The molecular basis of submergence tolerance during vegetative stage and deepwater rice. Under flash flooding stress (left), ethylene accumulation and perception was triggered by *SUB1A*. On the other hand, there is a feedback loop where accumulation of SUB1A transcripts will suppress the production of ethylene (Fukao et al. 2006). SUB1A maintains inhibition of GA-mediated growth responses directly through two GA-signaling repressor proteins, Slender Rice-1 (SLR1) and SLR Like-1 (SLRL1), or indirectly through the brassinosteroid (BR) pathway (Fukao and Bailey-Serres 2008; Schmitz et al. 2013). On the other hand, in deepwater rice (right), ethylene induces the transcription factors SNORKEL1 (SK1) and SNORKEL2 (SK2) that enhance GA-mediated internode elongation, keeping the rice plant's canopy above the water surface—an "escape" strategy (Hattori et al. 2009). This situation allows oxygen to be transported through aerenchyma to the submerged part of the plant

15.2.2 Adaptation to Stagnant Flooding and Rapid Elongation Ability

Even though breeding for stagnant flooding (SF) tolerance has long been one of the IRRI mainstream breeding programs for rainfed environments, thus far, there have been limited studies on this trait. Vergara et al. (2014) found wide variation in adaptation to SF among 626 rice accessions studied. Moderate elongation rate of 1.3–2.3 cm day^{-1}, as well as ability to produce more tillers, was optimum for adaptation to SF. Genetic studies have only recently been initiated (Singh et al. 2017). A recombinant inbred line population was derived from a cross of an improved high-yielding Indonesian variety, Ciherang-Sub1 (Septiningsih et al. 2015), and an IRRI elite breeding line, IR10F365, which is tolerant to both stagnant flooding and submergence during vegetative stage (Collard et al. 2013b). It was previously reported that the first generation of Sub1 varieties were susceptible to stagnant flooding (Vergara et al. 2014). The shorter the plant height of the Sub1 variety, the more susceptible it is to stagnant flooding. As an example, Swarna-Sub1, a short-stature variety, is very susceptible to stagnant flooding and is being used as a susceptible check for screening under stagnant flooding. A stagnant flooding-tolerant variety can be inherently tall or can be semidwarf but it elongates moderately with rising water to maintain its canopy above the water surface (Kato et al. 2014). Our results showed that Ciherang-sub1 was more tolerant to stagnant flooding stress compared to the first-generation Sub1 varieties. This tolerance was most likely due to its slightly tall stature; therefore even though its shoot elongation ratio and biomass are lower compared to the tolerant check, IRRI154 (NSIC Rc222), this variety still performed well under stagnant flooding. In general, grain yield was reduced by 50% due to stagnant flooding stress. Three yield QTLs and several QTL clusters for various traits that indirectly increase yield under stagnant flooding conditions were identified. The beneficial alleles of these QTLs were derived from both IR10F365 and Ciherang-Sub1 (Singh et al. 2017). Potential genes underlying these key QTLs can be further investigated to unravel the molecular mechanism underlying stagnant flooding tolerance. Furthermore, more QTLs can be mapped using new mapping populations derived from different donors—adding more targets for further physiological and molecular studies.

Deepwater and floating rice can be considered as special cases of stagnant flooding, where water depths remain above 50 cm for an appreciable part of the season. The main characteristic that distinguishes deepwater or floating rice varieties is the ability of the internodes (and shoots) to rapidly elongate when submerged. A number of QTLs for elongation ability have been identified (Hattori et al. 2007; Kawano et al. 2008; Nagai et al. 2012, 2014; Nemoto et al. 2004). Three QTLs identified by Hattori et al. (2007) were mapped on chromosomes 1, 3, and 12; the increased elongation alleles for all of them are derived from the deepwater rice variety C9285 (*Oryza sativa* ssp. *indica*). Based on comparative study of the location of the QTLs, the group also showed that the QTL on chromosome 12 is the major one for deepwater varieties (Hattori et al. 2008).

Through positional cloning, the chromosome 12 locus was found to consist of two ethylene responsive factor genes, *SNORKEL1 (SK1)* and *SNORKEL2 (SK2)*. These genes promote GA-mediated internode elongation—helping the rice plant to cope with stress by maintaining its canopy above the water surface to obtain oxygen and sunlight (Hattori et al. 2009). It was also observed that internode elongation was initiated at the sixth-leaf stage and that GA biosynthesis and signal transduction are essential for internode elongation in deepwater rice (Ayano et al. 2014). It is interesting to note that *SK1*, *SK2*, and *SUB1* are all ethylene-responsive transcription factors. As in the case of the non-SUB1 QTLs, there is also a probability that a few of the other elongation-ability QTLs could be involved in mechanisms that are completely different from *SK1* and *SK2*—this needs further study. A recent report demonstrated the role of *AtERF11* in promoting internode elongation through the ethylene biosynthesis inhibition and GA biosynthesis and signaling pathway activation. This gene is a member of the ERF subfamily VIII-B-1a of ERF/AP2 transcription factors in *Arabidobsis* (*Arabidopsis thaliana*) (Zhou et al. 2016). An ortholog of *AtERF11* that holds similar function may exist in rice. This needs further investigation. On a related issue, another recent report claimed that a novel rice protein family of OsHIGDs (hypoxia-induced gene domain), which are components of ethylene-independent hypoxia signaling, might be involved in early signaling of hypoxia-promoted stem growth in deepwater rice (Hwang and Choi 2016).

15.2.3 Submergence Tolerance at the Germination Stage: Anaerobic Germination

Tolerance of flooding during seed germination and very early seedling growth, referred to as anaerobic germination, is one of the most important traits necessary to ensure good seedling establishment in direct seeded rice (DSR) in both rainfed and irrigated ecosystems (Ismail et al. 2009). One of the most spectacular adaptive growth features of germinating rice seeds to tolerate oxygen deficiency in flooded soils is the accelerated growth of the coleoptile. Fast coleoptile elongation facilitates contact with air in waterlogged or flooded soils to maintain adequate aeration of the growing embryo. This accelerated elongation is independent of ethylene synthesis (Pearce et al. 1992), but is dependent on the extent of alcoholic fermentation and ethanol synthesis (Setter et al. 1994), which emphasizes the importance of anaerobic metabolism during germination and early seedling growth (Fig. 15.3). Several landraces with anaerobic germination (AG) tolerance have been identified (Angaji et al. 2010). Analysis of mapping populations derived from these tolerant genotypes has led to the identification of promising QTLs for molecular genetic studies and for use in breeding (Angaji et al. 2010; Baltazar et al. 2014; Septiningsih et al. 2013a, b). Additional AG QTLs have been identified from a biparental mapping population and genome-wide association studies (Hsu and Tung 2015;

Jiang et al. 2006; Zhang et al. 2017). Nonetheless, only one QTL, *qAG9-2* or *AG1*, has been fine-mapped thus far; the gene underlying the QTL, *OsTPP7*, was identified and functionally characterized. *OsTPP7* plays a role in increasing sink strength by partially alleviating feedback inhibition mediated by trehalose-6-phosphate (T6P) through increased T6P turnover. This leads to enhanced starch mobilization that generates more energy for accelerated coleoptile elongation to escape from anaerobic conditions (Fig. 15.3) (Angaji et al. 2010; Kretzschmar et al. 2015). Through over expression studies, Guo et al. (2016) demonstrated that the microRNA miR393a regulates coleoptile elongation and stomatal development

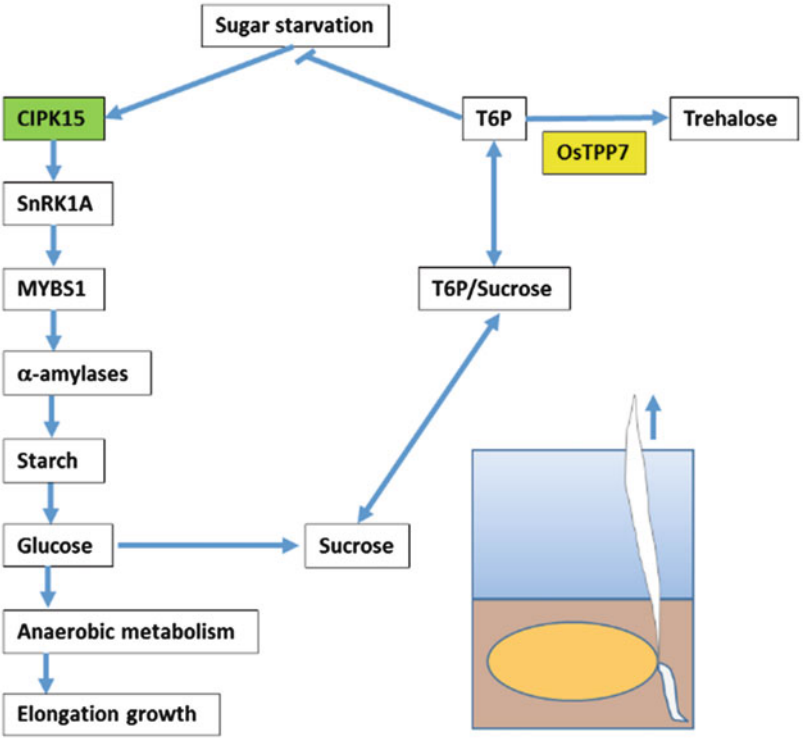

Fig. 15.3 The molecular basis of submergence tolerance during germination. Under hypoxia or anoxia, sugar starvation of an imbibed seed leads to a signaling cascade that eventually activates α-amylases through the involvement of a calcineurin B-like (CBL) protein, which targets the CBL interacting protein kinase 15 (CIPK15), which in turn triggers the SNF1-related protein kinase-1A (SnRK1A)-dependent sugar starvation-sensing cascade that induces MYBS1 transcription factor, a protein that directly binds to the promoter region of α-amylase genes (Lee et al. 2009). Only a small amount of energy is produced through glycolysis and alcoholic fermentation. Rice genotypes having functional OsTPP7 can enhance tolerance to anaerobic germination by partially alleviating feedback inhibition mediated by T6P level (Zhang et al. 2009) by indicating low sugar availability through increased T6P turnover. This leads to enhanced starch mobilization and subsequently enhanced growth of the germinating embryo and elongation of the coleoptile to facilitate timely escape from flooding (Kretzschmar et al. 2015)

via modulation of auxin signaling during seed germination and seedling establishment under anaerobic conditions. In another study, a candidate gene on chromosome 6, a DUF domain-containing protein (LOC_0s06g03520), which was highly induced by anaerobic germination, was recently claimed to be strongly associated with the trait (Zhang et al. 2017). However, further investigation is needed to better understand how these various mechanisms contribute to AG tolerance.

Unlike the *SUB1* gene that has a large phenotypic effect, major QTLs for anaerobic germination that have been identified so far have more moderate effects. However, it is assumed that different mechanisms of tolerance which are complementary can be combined to provide higher tolerance, in addition to the mechanism controlled by *OsTPP7*.

15.3 Breeding for Flood-Prone Environments

15.3.1 Flash Flood Environments

Breeding for submergence tolerance at IRRI started in the 1970s with large-scale screening of the international gene bank and identification of tolerant varieties (Vergara and Mazaredo 1975). Several of these varieties were used widely in crosses to transfer the trait into higher-yielding varieties (HilleRisLambers and Vergara 1982). The initially developed breeding lines had lower grain yields, but over several cycles of crossing and selection, some high-yielding lines were developed with strong submergence tolerance (Mackill et al. 1993). Several of them have been released in a few Asian countries. However, these were not widely adopted due to some undesirable traits, including poor grain quality and poor agronomic attributes. This problem arose due to linkage of submergence tolerance with the undesirable traits of the donor FR13A, despite its excellent tolerance to submergence.

The discovery and fine-mapping of the *SUB1* QTL, which largely contributes to submergence tolerance of FR13A (Xu and Mackill 1996; Xu et al. 2000, 2006), provided an opportunity for an accelerated and more precise breeding for submergence-tolerant rice. Closely linked and gene-based markers were developed to perform marker-assisted backcrossing (MABC) to convert susceptible mega varieties into tolerant lines without significantly altering beneficial traits of those superior varieties (Septiningsih et al. 2013a). In the beginning, IRRI successfully converted six mega varieties using two improved lines derived from FR13A, IR40931 and IR49830, as donors for submergence tolerance using the following mega varieties as recurrent parents: IR64 (Philippines); Swarna, Samba Mahsuri, and CR1009 or Savitri (India); BR11 (Bangladesh); and Tadokham 1 (TDK1; Laos) (Iftekharuddaula et al. 2011; Neeraja et al. 2007; Septiningsih et al. 2009). All six of these improved lines have been formally released in various Asian countries.

Later on, two "second-generation" Sub1 lines were developed at IRRI using IR64-Sub1 as the submergence-tolerant donor and Ciherang (Indonesia) and PSB

Fig. 15.4 Sub1 lines were grown under 25 days of complete submergence at the IRRI demonstration plot in 2014 dry season. The longer submergence duration was required because of lower than average temperature. Six pairs of the original and upgraded lines (IR64-Sub1, BR11-Sub1, PSB Rc18-Sub1, Swarna-Sub1, Ciherang-Sub1, and Samba Mahsuri-Sub1) were planted along with the susceptible check IR42. Varieties without Sub1 had high mortality

Rc18 (Philippines) as recurrent parents (Septiningsih et al. 2015). The last two improved lines were developed in a shorter time span than the former six Sub1 lines due to the use of a better donor, which was an elite variety and relatively higher genetic background similarity between donor and recipient varieties. The Sub1 lines showed a yield advantage of 2–3.5 t ha^{-1} under stress conditions compared to the intolerant parents (Singh et al. 2009) (Fig. 15.4). Most of the Sub1 lines developed through MABC in IRRI have been released in some countries in Southeast Asia and South Asia (Ismail et al. 2013; Mackill et al. 2012; Septiningsih et al. 2013a). Additionally, there have been several high-yielding lines that were conventionally bred with *SUB1* as well. The breeding line IR10F365 was developed for stagnant flooded environments (Collard et al. 2013b), but this line has also performed well in an irrigated trial in the Philippines (Gonzaga et al. 2017). Likewise, INPARA 3, which was selected and released by the Indonesian Center for Rice Research (ICRR), in Sukamandi, Indonesia, was developed for swampy areas; however, it was also found suitable for irrigated environments, especially the flood-prone regions.

The successful development of Sub1 varieties, especially Ciherang-Sub1, which was identified after only one generation of backcrossing and one generation of selfing (BC1F2), has been followed by the development of many other new submergence-tolerant lines using the elite Sub1 varieties, such as Swarna-Sub1

and BR11-Sub1, as donors of *SUB1* (Ahmed et al. 2016; Iftekharuddaula et al. 2016; Jena et al. 2015; Rao et al. 2016; Singh et al. 2016). With the discovery of additional QTLs complementary to *SUB1* (Gonzaga et al. 2016, 2017; Nandi et al. 1997; Septiningsih et al. 2012; Toojinda et al. 2003) and some new potential submergence-tolerant donors (Iftekharuddaula et al. 2015), it is expected that more robust-tolerant varieties can be bred to face the escalation of repeated and prolonged flash floods.

The released Sub1 varieties have been promoted to farmers in submergence-prone areas of South and Southeast Asia (Mackill et al. 2012). An estimated five million farmers have already adopted these varieties in South Asia (U. S. Singh, personal communication). The varieties have been evaluated in fields that are naturally submergence-prone in paired tests. For both Swarna-Sub1 and Samba Mahsuri-Sub1, average yield advantage was 1–1.5 t/ha in submergence-prone sites over their non-Sub1 parents in locations affected by submergence (Ismail et al. 2013).

15.3.2 Stagnant Flooded Environments

Breeding for stagnant flooding tolerance has been an important objective at IRRI and elsewhere for decades (Khush 1984; Mackill et al. 1996). Some criteria used to screen for tolerant genotypes have been yield, plant height, number of tillers, number of panicles, and tolerance to lodging under stagnant flooding. Among other traits, stagnant flooding tolerance is also routinely combined with *SUB1* to give additional protection in the event the plants are completely submerged, especially for rainfed lowland areas where flash floods often follow stagnant flooding events. In general, Sub1 varieties that are not too short to keep up with the raising water can survive well in this situation. A combination of bulk and pedigree breeding was used as the main method to develop new breeding lines (Fig. 15.5). Submergence screening is conducted in the F_2 and F_3 stages and again at a later stage (F_5/F_6) to make sure that there are no escapes.

Some elite breeding lines have been identified (Collard et al. 2013b; Mackill et al. 2010), and additional ones are in advanced testing. These lines have been sent to the IRRI partners, especially in South and Southeast Asia, to be tested in their affected areas. In India, for example, IRRI breeding lines together with breeding lines from the rice research centers in East India were used for selection activities among rice breeders in this region (Collard et al. 2013a). Similar activities have also been carried out in Bangladesh, Nepal, and some other countries in Southeast Asia.

Some of those advanced breeding lines are also being tested in swampy areas, such as in South Sumatra and South Kalimantan, Indonesia. In the swampy regions, however, varieties need to be also tolerant to other abiotic stresses, such as iron toxicity, aluminum toxicity, or salinity. Recently, collaboration between Indonesian and IRRI scientists identified several elite breeding lines that perform well in the swampy areas (Rumanti et al. 2016).

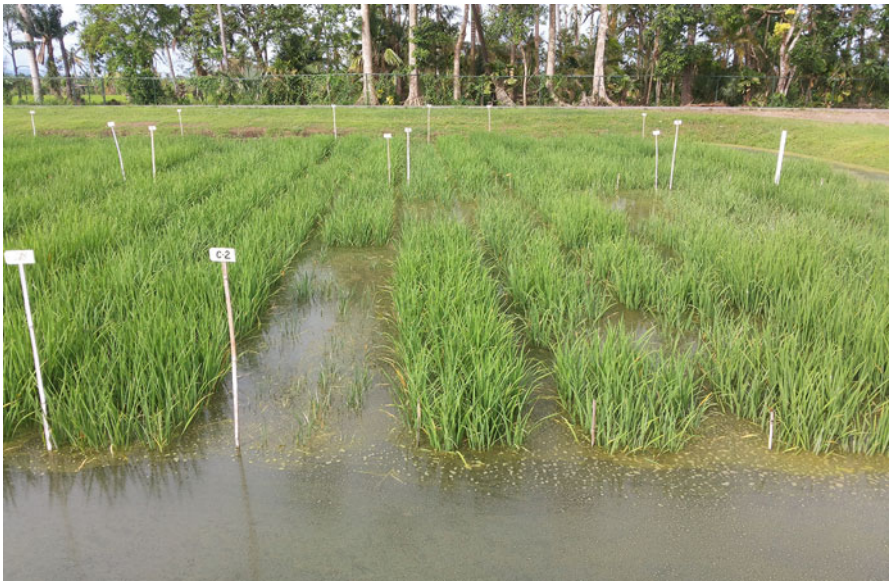

Fig. 15.5 Breeding line evaluation under stagnant flooding at the IRRI submergence field in 2014 wet season. Water was raised slowly every week starting 2 weeks after transplanting until water depth finally reached 50 cm during maximum tillering. This water depth was maintained until maturity. The plot that appears empty was Swarna-Sub1, the susceptible check. This variety almost completely died since it was too short to overcome the raising water

Nearly all deepwater and floating rice varieties are traditional landrace or pure-line selections from them. There has been virtually little or no impact of modern breeding for these areas despite the advances in genetic analysis cited above. This is not due to lack of effort. Rice breeders have been working on developing improved varieties for several decades (see Catling (1992) for a history of research on deepwater rice). Early work at IRRI focused on developing improved lines combining a high-yielding plant type of semidwarf or intermediate stature with strong elongation ability (Jackson et al. 1982). These lines often performed well in areas where flooding depths were not too extreme. A few improved, high-yielding elongating rice varieties were released, such as RD19 in Thailand (Prechachat et al. 1982).

Fewer plant breeders are now working on deepwater rice because of the decline of deepwater rice production in many countries. The main cause of this decline is the widespread introduction of tube-well irrigation for dry-season rice cultivation in low-lying areas. The farmers thus replace the lower-yielding deepwater crop with a very high-yielding dry season rice crop and leave the land fallow during the rainy season. However, the discovery of the SNORKEL genes *SK1* and *SK2* for rapid elongation has created new interest in developing varieties that can cope with deepwater flood levels (Hattori et al. 2009).

15.3.3 Direct Seeded Rice (DSR) and AG

Direct seeded rice (DSR) is increasingly popular in Asia mainly due to labor shortage (Kumar and Ladha 2011) and has long been the major crop establishment method in the Americas and Europe. AG tolerance is a useful trait where flooding can kill plants at the germination stage. In the IRRI breeding program, field screening for AG tolerance is conducted in F_2 stage under 5–7 cm water right after sowing. F_3 seeds harvested from surviving F_2 plants are subjected to the same screening, and seeds are harvested from selected F_3 survivors for further selection for other traits. A small number of $F_{5:6}$ seeds will be screened in concrete benches, and the tolerant lines will be further tested under AG field screening for confirmation (Fig. 15.6). High yield is one of the most important traits required for any DSR bred with AG tolerance. Therefore, in the past few years, the selected AG tolerance breeding lines are also entered in preliminary yield trials (PYT) under both irrigated and rainfed conditions. The selected lines from either or both environments then enter multilocation yield trials (MYT).

Some AG-tolerant lines with high grain yields and other beneficial traits, such as tolerance to submergence, have been selected. Although no yield penalty of this trait has been noted, this needs to be further tested under more conditions, including evaluation under more severe or prolonged flooding stress. Several of the AG-tolerant lines have other beneficial traits such as tolerance to Zn-deficient soil. Some of the advanced breeding lines have been sent to the IRRI partners in South and Southeast Asia and to several of the IRRI research hubs in India and Africa. Yield tests for all the tolerant lines are now underway to establish them as favorable varieties.

Marker-assisted breeding has been used to develop AG-tolerant lines by transferring major QTLs to some elite genetic backgrounds. *qAG-9-2*, a major QTL derived from Khao Hlan On (Angaji et al. 2010), referred to as *AG1*, was introgressed into several genetic backgrounds such as Ciherang-Sub1, IR64, IR64-Sub1, and IR64-Sub1+Saltol (Toledo et al. 2015). A preliminary yield trial showed no yield penalty or other negative traits due to the introgression *of AG1* (Toledo et al. 2015). Another QTL derived from Ma-Zhan Red, *qAG7.1*, referred as *AG2* (Septiningsih et al. 2013b), has been pyramided with *AG1* in some varieties, such as IR64-Sub1, Ciherang-Sub1, and PSB Rc82. Preliminary data from the IRRI experiment field station of those pyramided AG lines when combined with *SUB1* demonstrated that these improved lines showed high tolerance to AG and submergence during vegetative stage. The preliminary data from the IRRI field also showed that some of the best lines have significantly higher yield compared to the local checks (S. Dixit, personal communication). Both *AG1* and *AG2* have also been introgressed into popular varieties from the Philippines and Sri Lanka by partnering with PhilRice in the Philippines and the Rice Research and Development Institute (RRDI), Sri Lanka. With the discovery of more AG QTLs, it is expected that more AG-tolerant lines carrying combinations of several QTLs will be developed through marker-assisted breeding.

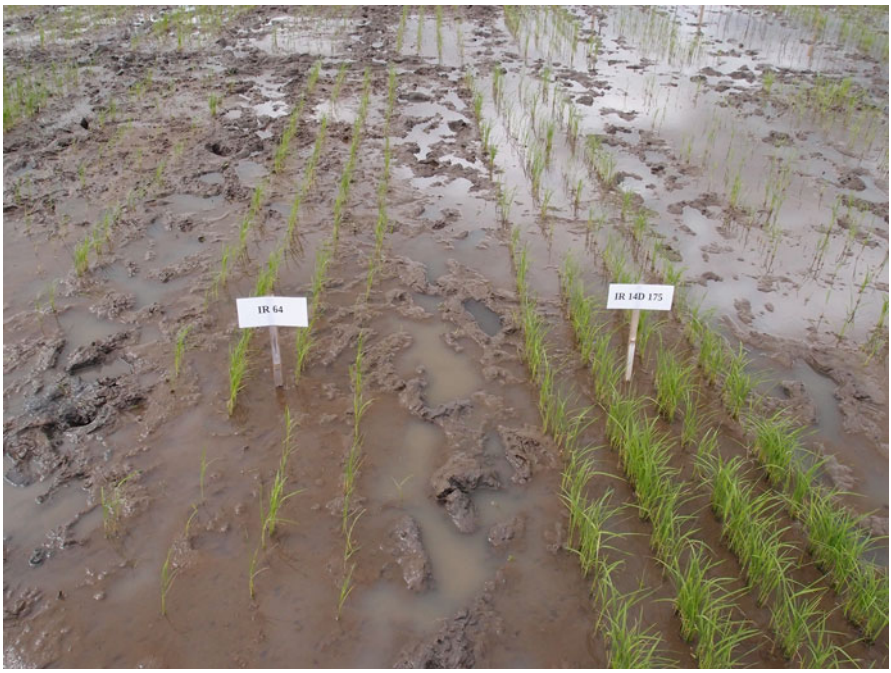

Fig. 15.6 Breeding line evaluation under anaerobic germination at the IRRI field in 2015 dry season. The field was flooded right after sowing at about 5 cm and was maintained for about 21 days to perform scoring. FR13A and IRRI154 can be used as susceptible checks; while some landraces, such as Khao Hlan On and Ma-Zhan Red, can be used as tolerant checks. Plot on the right having more survivors was IR14D175, one of the tolerant breeding lines; while plot on the left having fewer survivors was IR64, a popular high-yielding variety

15.4 Future Prospects for Flood-Tolerant Rice

There has been remarkable progress in developing breeding lines and varieties that combine tolerance to the different flooding stresses and other agronomic and grain quality traits. At this time, we can mainly document impact in flash flood environments due to the availability of Sub1 lines. Because of the MABC approach to rapidly transfer the *SUB1* gene into established varieties, the Sub1 lines have been quickly moved into production in flood-prone areas (Ismail et al. 2013; Mackill et al. 2012) and are providing benefits to rice farmers (Dar et al. 2013). Development of varieties suitable for medium-deep stagnant flooded areas has resulted in some success as well (Mackill et al. 1996; Reddy et al. 2013), and many new breeding lines developed for these conditions are under evaluation. The new breeding lines with the AG trait need further evaluation. It is not yet clear how the SNORKEL genes for rapid elongation will be utilized to develop varieties suitable for deeper flooded conditions. Determining how these genes perform in different genetic backgrounds will be important to decide which conditions they

will be appropriate in. The most promising situations will be where water depths are not too extreme and higher yields can be attained. In these areas, moderate elongation may be more desirable to avoid extensive lodging after floods recede.

Many of the tolerant lines developed through both conventional and molecular breeding strategies have been used by rice breeders at IRRI and national partner institutes as parents to further develop higher-yielding varieties that are tolerant to different flooding scenarios and that could adapt well to the local environments. One example of major efforts in molecular breeding to incorporate different QTLs for submergence, drought, and salinity tolerance traits was demonstrated by Singh et al. (2016).

There are also some major molecular breeding efforts at IRRI to develop high-yielding resilient improved lines by combining several key traits, including tolerance to submergence and drought, submergence and salinity, and submergence and resistance to several diseases. These improved lines have been tested in several target regions. In the future, with the identification of more major QTLs for different flooding traits as well as other relevant traits, improved lines can be developed using some of those QTL targets to further improve the existing elite lines to develop resilient rice varieties under different stress scenarios. These novel major QTLs also open new doors to further investigate molecular mechanisms underlying these flooding tolerance traits, including tolerance to stagnant flooding—a trait that is still poorly understood.

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Chapter 16
The Gene Network That Regulates Salt Tolerance in Rice

Dai-Yin Chao and Hong-Xuan Lin

Abstract Rice is one of the glycophytes and its yield and grain quality is threatened by salinity. During the past decades, great progresses have been made on molecular mechanisms of rice dealing with salt stress. Taking advantage of genetics, transcriptome studies, and forward genetics, hundreds of genes involved in salt tolerance or salt stress response have been identified. According to their functions, these genes could be divided into at least three types: signaling components, transcriptional factors, and downstream functional molecules including transporters, enzymes for compatible solute synthesis, and ROS scavengers. Based on these knowledges and those obtained from *Arabidopsis thaliana*, this review summarizes these findings and tries to draw a rough picture of the gene networks controlling salt tolerance of rice.

Keywords Rice · Salt tolerance · Gene network · Ion homeostasis · Osmotic adjusting solutes · Transporters · ROS scavengers · Transcriptional factors · Signaling components

16.1 Introduction

The salinization of irrigated farmland is becoming increasingly detrimental to plant biomass production and agricultural productivity, because most plant species are sensitive to high concentrations of Na^+. Rice (*Oryza sativa*) is a glycophyte (as opposed to a halophyte), and as little as 30 mM NaCl (electronical conductivity,~2–3 dSm^{-1}) is able to significantly inhibit the growth of rice seedlings. Salt stress affects plant growth and development mainly by (1) disturbing ion homeostasis and (2) by producing osmotic stress. However, plants respond to salt stress

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through various biological processes, such as stomatal movement, osmotic adjustment, ion transporter activation, cell wall modification, protection of the photosynthetic reaction centers, ROS scavenging, and developmental reprograming. These processes involve a large number of functional genes, as well as many transcription factors (TFs) and signaling components. Many of these genes have been identified and characterized over the past two decades, providing insight into the primary framework of the gene networks that regulate plant salt tolerance. Although this knowledge was mainly acquired from studies on *Arabidopsis thaliana,* progress has also been made in the most important model crop species, rice, using both forward and reverse genetics approaches. In this chapter, we will summarize the current understanding of the gene networks involved in salt tolerance in rice. Based on their functions, the salt tolerance-related genes can be divided into three groups: (1) downstream functional genes such as transporters, enzymes that catalyze the synthesis of osmotic adjusting solutes, and ROS scavengers, (2) TFs that reprogram the rice transcriptome in response to salt stress, and (3) signaling components that activate the functions of downstream genes and TFs. We will attempt to draw a conceptual picture of the gene networks that control salt tolerance in rice based on research progress made in recent decades (Fig. 16.1).

16.2 Genes Responsible for Ion Homeostasis Under Ionic Stress

High concentrations of Na^+ not only change the cytoplasmic ion strength that is essential for cellular metabolism but also disturb the homeostasis of other mineral elements such as K^+. Therefore, limiting the entrance of Na^+ into the cell cytoplasm or compartmentalization of Na^+ in the vacuole is required for salt tolerance in plants. Furthermore, different cell types and tissues play different roles during the plant life cycle and are distinctively sensitive to ionic stress. Thus, the distribution of Na^+ in different cell types and tissues is also regulated for better adaptation to salt stress. For example, the shoot is more sensitive to salt than is the root, and limiting root-to-shoot transport of Na^+ is an important mechanism for salt tolerance in rice. Several Na^+ transporters have been identified as being responsible for Na^+ homeostasis during these processes.

The vacuolar sequestration of Na^+ is mainly mediated by a type of Na^+/H^+ antiporter localized on the tonoplast. The role of this type of transporter in plant salt tolerance was revealed by overexpression and knockout of the *NA*$^+$*/H*$^+$ *EXCHANGER 1* (*AtNHX1*) gene in *Arabidopsis thaliana* (Apse et al. 1999, 2003). There are five NHX-type Na^+/H^+ antiporter genes in the rice genome that are inducible by salt stress (Fukuda et al. 2011). Complementation in yeast confirmed that at least four of these function as Na^+/H^+ antiporters, and overexpression of *OsNHX1* was shown to enhance salt tolerance in rice (Amin et al. 2016; Fukuda et al. 2004, 2011). Interestingly, these genes are differentially expressed in different

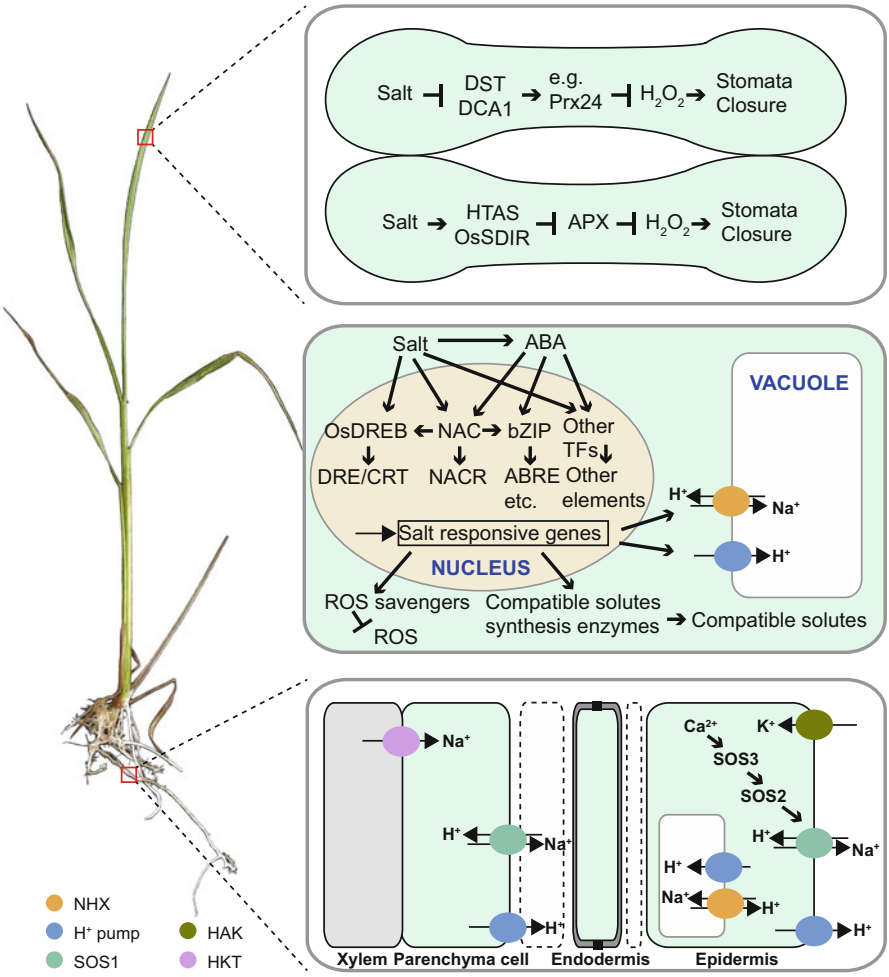

Fig. 16.1 The gene networks controlling salt tolerance of rice. The transporters are presented by ovals with different colors as indicated at the lower left corner

tissues and cell types, suggesting that the rice NHX genes play important roles in salt tolerance that differ depending on the cell type.

The other type of Na^+/H^+ antiporter, *SALT OVERLY SENSITIVE 1* (*SOS1*), also plays an essential role in plant salt tolerance by mediating Na^+ redistribution at the cellular level. The *SOS1* gene was first identified in *A. thaliana* by screening for mutants that were overly sensitive to salt stress (Shi et al. 2000). The SOS1 protein is localized on the plasma membrane (PM) of the root tip epidermis cells and xylem parenchyma cells and functions in the efflux of Na^+ into the rhizosphere and apoplastic spaces of the roots under salt stress (Shi et al. 2002). The rice genome also contains a homolog of *SOS1*, which was named *OsSOS1*. Similar to

SOS1, OsSOS1 functions in the efflux of Na^+, indicating that rice and *A. thaliana* share a common mechanism mediated by SOS1.

Whether they are located on the tonoplast or the PM, Na^+/H^+ antiporters require a H^+ gradient to drive the efflux of cytosolic Na^+. Proton pumps are therefore important in salt tolerance. Overexpression of proton pumps on either the tonoplast or PM will enhance plant salt tolerance (Gaxiola et al. 1999, 2001; Gevaudant et al. 2007), while reduced H^+-ATPase activity results in hypersensitivity to salt stress (Hubbs et al. 2007). In rice, activities of both tonoplast and PM proton pumps were observed to be upregulated by salt treatment, suggesting that the H^+-ATPase genes also play a role in rice salt tolerance (Pons et al. 2011; Shen et al. 2011).

While the Na^+/H^+ antiporters that mediate exclusion or compartmentalization of Na^+ are very important in salt tolerance, a type of protein known as the high-affinity K^+ transporter (HKT) was also found to be essential for salt tolerance in plants because it controls the Na^+ distribution between different tissues. The HKT1 protein was initially identified to function as a Na^+/K^+ cotransporter in wheat (*Triticum aestivum*) (Schachtman and Schroeder 1994). Its homologs *AtHKT1;1* and *SKC1*/*OsHKT1;5* were then shown to control natural variation in shoot Na^+ content and salt tolerance in *A. thaliana* and rice, respectively (Ren et al. 2005; Rus et al. 2006). Unlike TaHKT1, however, both AtHKT1;1 and OsHKT1;5 only transport Na^+. These genes are predominantly expressed in root xylem parenchyma cells and function in retrieving Na^+ from the transpiration stream (Ren et al. 2005; Sunarpi et al. 2005). Interestingly, the *SKC1* gene was cloned via a QTL analysis of shoot K^+ content under salt stress, indicating that *SKC1*/*OsHKT1;5* indirectly affects K^+ homeostasis (Ren et al. 2005). A similar phenotype was also observed in the null mutant *athkt1-1* (Sunarpi et al. 2005), suggesting that the K^+ deficiency caused by increased Na^+ is a disastrous effect of salt stress.

Unlike *A. thaliana*, which has only a single *HKT* gene, *AtHKT1;1*, the rice genome contains nine *HKT* genes (including one pseudogene). Based on protein sequence alignment, these nine genes can be divided into two subfamilies. The most important difference between the two subfamilies is a glycine/serine change in the first pore loop, because it determines the ion selectivity of HKT proteins (Garciadeblas et al. 2003; Maser et al. 2002). The members of Subfamily 1 have a serine at this position and function as specific Na^+ transporters, while the members of Subfamily 2 have a glycine at the same position and function as Na^+-K^+ cotransporters. Both AtHKT1;1 and OsHKT1;5 are members of Subfamily 1, and other Subfamily 1 members in rice have also been found to play a role in salt tolerance. *OsHKT1;4* is the closest homolog of *OsHKT1;5* in the rice genome. Knocking down the expression of *OsHKT1;4* demonstrated that this gene is involved in salt tolerance at the reproductive stage because it functions in Na^+ unloading in the xylem of leaf sheaths and stems (Suzuki et al. 2016). The other *HKT* family member, *OsHKT1;1*, is also required for salt tolerance in rice, possibly by mediating recirculation of Na^+ in the phloem as well as unloading Na^+ from the xylem (Wang et al. 2015). Recently, a genome-wide association study (GWAS) of rice salt tolerance identified a locus that controls root Na^+ content on chromosome 4, which was then named *Root* Na^+ *Content 4* (*RNC4*). Both *OsHKT1;4* and

OsHKT1;1 localize to the candidate region of *RNC4*, and further evidence confirmed that *OsHKT1;1*, but not *OsHKT1;4*, drives the natural variation in root Na^+ content in rice during salt stress (Campbell et al. 2017). This finding is consistent with the role of *OsHKT1;1* in Na^+ recirculation.

The range of substrates utilized by Subfamily 2 HKT proteins is wider than that of Subfamily 1. In addition to Na^+, members of HKT Subfamily 2 also can transport K^+ and Ca^{2+} (Garciadeblas et al. 2003; Lan et al. 2010). OsHKT2;1, a Subfamily 2 HKT protein, mediates Na^+ uptake in the rice root when exposed to conditions of K^+ starvation and was found to be rapidly downregulated in response to salt stress (Horie et al. 2007), indicating that OsHKT2;1 plays a negative role in salt tolerance in rice. Similarly, OsHKT2;2 also mediates the influx of K^+ and Na^+, and the induction of *OsHKT2;2* in the salt-tolerant rice variety "Nona Bokara" was observed to be suppressed by salt treatment, suggesting that *OsHKT2;2* also plays a role in rice salt tolerance.

One of the toxic effects of Na^+ is to disturb homeostasis of other elements, especially K^+. Therefore, other metal transporters and channels are also involved in salt tolerance in rice. The *Oryza sativa* contains 27 members of high-affinity K^+ transporters (HAKs), and at least OsHAK1, OsHAK5, and OsHAK21 have found to mediate specific K^+ influx and are inducible in response to salt stress (Chen et al. 2015; Shen et al. 2015; Yang et al. 2009, 2014). Reverse genetic analysis established that *OsHAK1*, *OsHAK5*, and *OsHAK21* all play important roles in rice salt tolerance by activating K^+ uptake, thus maintaining high K^+/Na^+ ratios under salt stress conditions (Shen et al. 2015; Yang et al. 2014). Recently, a magnesium transporter OsMGT1 was also found to be required for salt tolerance in rice (Chen et al. 2017), possibly by regulating the transporter activity of OsHKT1;5.

In addition to specific transporters, the Casparian strips and suberin lamellae of the cell exo- and endodermis also play essential roles in preventing water loss and limiting the entrance of Na^+ into the transpiration stream. A recent study showed that high concentrations of Na^+ can enhance the disposition of suberin through activation of the ABA pathway (Barberon et al. 2016). Though little is known about the gene network regulating Casparian strips and suberin lamellae in rice in response to salt stress, the bypass flow of Na^+ via the apoplastic pathway has long been known to be a significant contribution of shoot Na^+ accumulation to salt stress (Horie et al. 2012; Krishnamurthy et al. 2009; Yeo et al. 1987). Rice homologs of the genes that regulate Casparian strips and suberin lamellae in *A. thaliana* might be involved in limiting apoplastic Na^+ bypass and salt tolerance in rice.

16.3 Genes Involved in Osmotic Adjustment

In addition to ionic stress, high concentrations of Na^+ also cause severe osmotic stress in plants. To deal with osmotic stress, plants can activate at least three mechanisms, including adjusting water uptake, synthesizing compatible solutes, and preventing water loss. The most efficient means of water uptake is through

water channel aquaporins. However, when high concentrations of Na^+ are present on the root surface, the extracellular osmotic potential (ψ_{osm}) can be lower than the cellular ψ_{osm} and could immediately trigger water efflux from the roots. Therefore, one of the rapid responses of the root is to shut down the water channels, a phenomenon that is widely observed in *A. thaliana*, corn, and barley. This process can be completed at the transcriptional level or the posttranscriptional level and can include protein modification (Horie et al. 2011) and subcellular redistribution (Boursiac et al. 2005, 2008; Maurel et al. 2008).

The rice genome contains 33 aquaporin genes, and several of them, such as *OsPIP2;3*, *OsPIP2;5*, and *OstTIP2;1*, have been shown to be predominantly expressed in the root (Sakurai-Ishikawa et al. 2011; Sakurai et al. 2005, 2008). Similar to the observations in *A. thaliana,* corn, and barley, a series of *OsPIP* and *OsTIP* genes were also found to be downregulated by salt stress in rice (Guo et al. 2006; Kawasaki et al. 2001). These results suggest that aquaporin genes might also play important roles in salt stress.

Accumulation of osmotic adjustment solutes helps decrease the cellular ψ_{osm} and thus promotes water influx under conditions of salt stress. A group of organic compounds, such as proline, glycinebetaine, trehalose, and polyols (such as myo-inositol), are synthesized by plants, especially some species from extreme environments, during salt and drought stress (Bohnert et al. 1995; Hasegawa et al. 2000). These nontoxic organic compounds are called compatible solutes, and they reduce the cellular ψ_{osm} and protect protein activities during osmotic stress. Considerable efforts have been directed toward the application of these compatible solutes or the expression of exogenous enzymes producing these solutes to improve salt tolerance in rice (Garg et al. 2002; Sobahan et al. 2009); however, there is very little evidence to show that native rice genes are involved in the synthesis of compatible solutes.

Transcriptome studies of rice under salt stress revealed that quite a few of the genes involved in trehalose synthesis are upregulated (Chao et al. 2005; Kawasaki et al. 2001), which is consistent with the observation that trehalose accumulates in salt-stressed rice roots (Garcia et al. 1997). Overexpression of either *OsTPP1* or *OsTPS1*, two genes encoding enzymes for trehalose synthesis, can promote salt tolerance in rice (Ge et al. 2008; Li et al. 2011). However, the trehalose does not accumulate to high enough levels to function as a compatible solute either in *OsTPP1* or *OsTPS1* overexpressing plants, and it was thus hypothesized to be a signal component in the salt response or to play a regulatory role in carbon metabolism under salt stress (Ge et al. 2008; Li et al. 2011).

Another compatible solute, myoinositol, was found to play an important role in a halophytic wild rice, *Porteresia coarctata* (also known as *Oryza coarctata*), in adaptation to salt stress (Sengupta and Majumder 2010). Expression of the *P. coarctata L-myo-inositol-1-phosphate synthase* (*PcINO1*) gene remarkably enhanced salt tolerance in tobacco, indicating that genes involved in synthesis of myoinositol and its derivates are vital for salt tolerance in this species (Das-Chatterjee et al. 2006; Ghosh Dastidar et al. 2006; Majee et al. 2004). Rice cultivars also have *INO1* genes, but their roles in salt tolerance are unclear at present (Ray et al. 2010).

Endodermis suberization and stomatal closure are two major mechanisms by which plants prevent water loss during salt stress. As mentioned above, the molecular basis for endodermal suberization of rice in response to salt stress remains unknown. Stomatal movement is controlled by a complicated gene network, and the genes involved in salt-stressed stomatal closure are described below.

16.4 Genes Involved in Stomatal Movement During Salt Stress

Stomatal closure in response to salt stress is mediated by the phytohormone ABA and reactive oxygen species (ROS), which function as signaling molecules (Song et al. 2014). Some of the genes involved in this process have been characterized in rice. The RING-finger ubiquitin E3 ligase-encoding gene *HEAT TOLERANCE AT SEEDLING STAGE* (*HTAS*) is inducible by ABA and multiple abiotic stresses including salt stress; overexpression of this gene enhances stomatal closure, while knockdown of *HTAS* expression inhibits stomatal closure (Liu et al. 2016). Further study indicated that *HTAS* might mediate the degradation of an ascorbate peroxidase, thus promoting ROS-mediated stomatal closure (Liu et al. 2016). Similarly, another salt-inducible RING-finger E3 ligase, *Oryza sativa* SALT-AND DROUGHT-INDUCED RING FINGER 1 (OsSDIR1), was also found to be involved in stomatal closure during salt stress, because overexpression of *OsSDIR1* enhanced stomatal closure and salt tolerance in rice (Gao et al. 2011). Although the molecular mechanisms underlying the involvement of *OsSDIR1* in stomatal movement require further investigation, studies on its homolog in *A. thaliana* revealed that *SDIR1* activates the ABA pathway through its target gene *SDIRIP1* (*SDIR-INTERACTING PROTEIN 1*) (Zhang et al. 2015).

A well-established stomatal movement pathway found in rice is the DST-mediated pathway. *DST* (*Drought and Salt tolerance*) encodes a zinc finger type of TF, and mutation of this gene improves drought and salt tolerance in rice (Huang et al. 2009). Further study showed that DST can bind to the promoters of a series of genes encoding ROS scavengers such as peroxidase 24 and then activate them. When exposed to salt or drought stress, expression of *DST* in guard cells is downregulated within 30 min. This suppression of *DST* expression reduces the levels of ROS scavengers and allows H_2O_2 to accumulate, subsequently resulting in stomatal closure (Huang et al. 2009). This process requires not only DST but also its partner DCA1 (DST coactivator). DST and DCA1 form a heterologous tetramer which promotes the expression of peroxidase 24 (Cui et al. 2015). In addition to peroxidase 24, a leucine-rich repeat (LRR)-RLK gene named *Leaf Panicle 2* (*LP2*) was also found to be downstream of *DST*. *LP2* is also required for stomatal closure in response to salt stress by regulating H_2O_2 (Wu et al. 2015a), indicating that *DST* controls H_2O_2 levels via multiple mechanisms.

16.5 Genes Involved in ROS Scavenging

ROS are important signaling components in stomatal movement as well as in some other physiological processes, but they are also deleterious to many essential molecules, such as nucleic acids, proteins, membrane lipids, and components of the photosynthetic apparatus. The production of ROS is induced by biotic and abiotic stresses, and their levels need to be well-regulated. To cope with this, plants have evolved complicated gene networks, including those encoding peroxidase (POD), superoxide dismutase (SOD), catalase, dehydroascorbate reductase (DHAR), and a series of proteins involved in catalyzing and regulating the synthesis of antioxidants.

As in other abiotic stresses, salt stress also enhances the production of ROS. Many independent studies have identified a series of salt tolerance-related genes that function in ROS scavenging in rice. Ascorbic acid is an important cellular antioxidant, and the synthesis of ascorbic acid in plants involves the enzyme GDP-D-mannose pyrophosphorylase (GMPase) (Conklin et al. 1999). A recent study showed that knockdown of the GMPase gene *OsVTC1-1* in rice resulted in the overaccumulation of ROS and a decrease in salt tolerance (Qin et al. 2016). Interestingly, a monocot-specific microRNA, miRNA528, that targets a gene encoding ascorbic acid oxidase (AAO), was also found to be required for salt tolerance in rice (Yuan et al. 2015). Overexpression of miRNA528 reduced AAO activity and improved salt tolerance in rice, indicating the involvement of miRNA528 in salt tolerance through its regulation of *AAO* expression (Yuan et al. 2015).

Glutathione is another important antioxidant in plants. Transcriptome studies have indicated that the expression of several genes related to glutathione metabolism is reprogramed during salt stress (Chao et al. 2005; Kawasaki et al. 2001; Rabbani et al. 2003; Walia et al. 2005). Reverse genetics showed that a mitochondrial glutathione peroxidase, GPX1, is required for salt tolerance in rice, and knockdown of this gene induces accumulation of ROS that impairs photosynthesis during salt stress (Lima-Melo et al. 2016). The mitochondrion is an important source of ROS, and the genes required for maintenance of mitochondrial functions are thus also important in salt tolerance. It has been reported that overexpression of the fertility restorer gene *Rf5* can improve salt tolerance in transgenic rice plants, because the Rf5 protein can suppress translation of the *orfh79* mRNA, thus restoring mitochondrial function that was impaired by the accumulation of ORFH79, a cytoplasmic male sterility protein (Yu et al. 2015). Peroxisomes are as important as mitochondria in the production of ROS and thus play a vital role in a number of biological processes, including the abiotic stress response. A peroxisomal biogenesis factor, OsPEX1, was recently reported to be associated with salt tolerance in rice, because overexpression of *OsPEX1* activates antioxidant enzymes and decreases lipid peroxidation during salt stress (Cui et al. 2016). In addition to mitochondria and peroxisomes, plasma membrane NADPH oxidases also play a key role in the production of ROS under stress conditions. *OsRbohA*, a rice plasma

membrane NADPH oxidase, was recently shown to mediate ROS regulation and tolerance to abiotic stresses including high salt levels (Wang et al. 2016).

Several other genes related to ROS-scavenging have also been reported to contribute to salt tolerance in rice, but detailed descriptions of their actions will require further investigation. For example, some rice jasmonic acid (JA) biosynthesis mutants were shown to be salt tolerant. Further analysis showed that these JA mutants were impaired in allene oxide cyclase (AOC) function, which improved ROS scavenging and promoted salt tolerance (Hazman et al. 2015). Interestingly, two DNA/RNA helicases, OsSUV3 and PDH45, were shown to be involved in salt tolerance and the regulation of ROS homeostasis in rice, but the underlying mechanism remains unclear (Nath et al. 2013, 2016). In addition, a rice cyclophilin gene, *OsCYP2*, also acts as a regulator of ROS homeostasis and contributes to salt tolerance in rice, but an understanding of its mode of action will require further investigation (Ruan et al. 2011).

16.6 TFs Involved in Salt Tolerance in Rice

When exposed to salt stress, plants have to alter their development to adapt to the new environment. This process requires transcriptome reprogramming that is initiated by a series of TFs. In the past 15–20 years, a milestone achieved in the field of abiotic stress research was the discovery and characterization of the DREB (DRE-binding protein)/CBF (DRE/CRT-binding factor)-mediated regulatory pathway. Early studies revealed that many of the genes induced by abiotic stresses contain a DRE (dehydration-responsive element)/CRT (C-repeat) element with a core sequence A/GCCGAC (Yamaguchi-Shinozaki and Shinozaki 1994). Two groups independently identified the TFs that target the DRE/CRT cis-element by using yeast one-hybrid assays and found that they belong to a large family of AP2 (APETALA)/ERF (ethylene-responsive element-binding) TFs (Liu et al. 1998). These TFs are rapidly induced and activated by different abiotic stresses, and they trigger the expression of downstream genes containing the DRE/CRT element, such as *LEA* (Late-Embryogenesis Abundant) and *COR* (cold-responsive) genes. In addition, subsequent research has identified additional genes in the CBF/DREB pathway, such as *ICE1* (Inducer of CBF expression 1) and *CAMTA1* (calmodulin-binding transcription activator) (Chinnusamy et al. 2003; Pandey et al. 2013).

Based on sequence analysis, the rice genome contains at least ten *DREB1*-type genes and four *DREB2*-type genes. Overexpression of *OsDREB1A* and *OsDREB1B* either in *A. thaliana* or rice enhances salt tolerance in the transgenic plants (Dubouzet et al. 2003; Ito et al. 2006). Interestingly, the retarded growth phenotype caused by the overexpression of *AtDREB1* was also observed in plants overexpressing *OsDREB1A* and *OsDREB1B*. In addition, the target genes of *AtDREB1* and *OsDREB1A* were found to largely overlap, providing evidence for the functional conservation of *DREB1* in *A. thaliana* and rice. Overexpression of

another two salt inducible DREB1-type genes, *OsDREB1F* and *OsDREB1G*, was also shown to improve salt and/or drought tolerance in rice and *A. thaliana* by activating downstream genes containing DRE/CRT elements (Chen et al. 2008; Wang et al. 2008). Similarly, the OsDREB2-type genes *OsDREB2A* and *OsDREB2B* are also induced by abiotic stresses including high salinity (Matsukura et al. 2010). Overexpression of *OsDREB2B* in either rice or *A. thaliana* improved osmotic stress tolerance, suggesting that it might be also involved in the salt stress response (Chen et al. 2008; Matsukura et al. 2010). In addition to DREB/CBF TFs that bind DRE/CRT elements, other AP2/ERF-type TFs, such as AP37 (Oh et al. 2009) and OsERF922 (Liu et al. 2012), also act as regulators of salt responsive genes.

The ABRE (ABA response element) that contains the core sequence ACGT is another well-known cis-element that is present in the promoters of stress- and ABA-induced genes (Guiltinan et al. 1990). The TFs that bind to the ABRE were identified as a group of bZIP (basic leucine zipper)-type proteins and were named AREB (ABRE-binding protein) or ABF (ABRE-binding factor) (Choi et al. 2000; Uno et al. 2000). Both ABRE and AREB/ABF proteins are important for transcriptome reprograming in response to abiotic stresses including high salt conditions. Many *AREB*/*ABF*-homologous genes are also regulated by salt stress and contribute to salt tolerance in rice. For example, knocking out the salt-inducible rice gene *OsABF2* resulted in a hypersensitive phenotype to salt stress (Hossain et al. 2010). Some other bZIP-type TFs related to the salt stress response and salt tolerance were also identified in rice. *OsbZIP23*, *OsABL1*, and *OsABI5* are three *bZIP* genes that are all induced in response to salt stress. Overexpression of *OsbZIP23* and *OsABL1* promotes salt tolerance, while overexpression of *OsABI5* suppresses salt tolerance in rice (Xiang et al. 2008; Yang et al. 2011; Zou et al. 2008), suggesting that functional differentiation exists among the different bZIP TFs. Interestingly, an extensive analysis of gene expression in 89 rice bZIP TFs showed that at least 37 of them are responsive to abiotic stresses (Nijhawan et al. 2008), indicating that bZIP family TFs are widely involved in abiotic stresses.

In addition to the DREB/CBF and bZIP regulons, the NAC (NAM, ATAF, and CUC)-type TFs are also essential for transcriptome reprograming during salt stress. Using yeast one-hybrid assays, Tran et al. (2004) isolated three NAC-type TFs (ANAC019, ANAC055, and ANAC072) drive expression of the salt- and drought-inducible gene *ERD1*. Further transcriptomic analyses of the transgenic plants overexpressing the *NAC* genes confirmed that these types of TFs control the expression of many salt- and drought-responsive genes (Tran et al. 2004). Soon after the identification of the NAC regulon in *A. thaliana*, the rice NAC homolog SNAC1 was also found to play an important role in rice salt tolerance. *SNAC1* is inducible by numerous abiotic stresses including salt, and the overexpression of *SNAC1* significantly improved salt and drought tolerance in rice without any visible adverse effects (Hu et al. 2006). In addition to *SNAC1*, many other *NAC* genes have been shown to be induced by salt stress and to be involved in salt tolerance in rice.

These genes include *SNAC2*/*OsNAC6* (Hu et al. 2008; Nakashima et al. 2007), *OsNAC5* (Takasaki et al. 2010), *OsNAC10* (Jeong et al. 2010), *ONAC106* (Sakuraba et al. 2015), *ONAC022* (Hong et al. 2016), *OsNAC2* (Shen et al. 2017), and *ONAC045* (Zheng et al. 2009).

Overexpression of these *NAC* genes is useful not only for improving rice salt tolerance but also provides genetic tools for studying downstream genes. For example, based on transcriptome changes in the overexpressing plants, *ONAC106* was demonstrated to induce expression of *OsNAC5*, *OsDREB2A*, *OsbZIP23*, and *OsLEA3* (Sakuraba et al. 2015); *ONAC022* also targets *OsDREB2A*, *OsLEA3*, and *OsbZIP23*, as well as other TF- and stress-responsive genes (Hong et al. 2016); and *OsNAC2* was found to mainly regulate ABA-dependent stress-responsive genes (Shen et al. 2017).

The DREB/CBF, bZIP, and NAC are three major groups of TFs that are involved in transcriptome reprogramming in rice exposed to salt stress. However, many other types of TFs also play roles in this process. These TFs belong to nine protein families: zinc finger, MYB, ARR-B, TCP, CPP, NIN-like, bHLH, WRKY, and TIFY. The salt tolerance-related TFs in the zinc finger family include DST, ZFP252, ZFP179, and ZFP185. DST was discussed above concerning its role in stomatal movement. ZFP252 was demonstrated to trigger expression of *OsDREB1A*, and its overexpression increased salt stress tolerance in rice (Xu et al. 2008). Overexpression of *ZFP179* promoted the accumulation of compatible solutes, such as proline and soluble sugars, and thus improved salt tolerance in rice (Sun et al. 2010). ZFP185 is an A20-/AN1-type zinc finger protein that is involved in crosstalk between the GA and ABA signaling pathways, but it lacks transcription activation potential and plays a negative role in the response to abiotic stresses (Zhang et al. 2016). The MYB-type TF OsMYB3R-2 was shown to increase the expression of *OsDREB2A* and to improve cold, drought, and salt tolerance in rice (Dai et al. 2007), while the R2R3-type MYB protein OsMYB91 regulates expression of *SLR1*, a rice homolog of DELLA genes required for coordinating plant growth and abiotic stresses, to control salt tolerance in rice (Zhu et al. 2015). Interestingly, the expression of *OsHKT1;1* is also controlled by another MYB-type TF, OsMYBc, which probably functions together with the ARR-B-type TF OsRR22 (Ismail and Horie 2017). It has been shown that OsMYBc is able to bind the AAANATNY motif in the *OsHKT1;1* promoter, and loss-of-function of *OsMYBc* results in decreased salt tolerance in rice and a reduced level of expression of *OsHKT1;1* under salt stress (Wang et al. 2015). In contrast to *OsHKT1;1*, the Na^+/H^+ transporter gene *OsNHX1* is regulated at the transcriptional level by five TFs that belong to the TCP-, CPP-, and NIN-like protein families (Almeida et al. 2017). The transcription factor that controls another salt tolerance-related ion transporter, OsHAK21, was identified as the bHLH-type TF OsbHLH062 (Wu et al. 2015b). The roles of stress-induced TFs, including the WRKY-type OsWRKY45 and the TIFY-type OsTIFY11a, were also characterized by reverse genetics (Tao et al. 2011; Ye et al. 2009).

16.7 Rice Genes Involved in Salt Response Signaling

The first step of a plant's response to salt stress is to perceive and translate the Na^+ and osmotic signals into a cascade of biochemical reactions that alter the cell status at the transcriptional and posttranscriptional levels to adapt to the environmental change. Although the identities of the sensors that perceive Na^+ and osmotic stress remain elusive, some signaling components in this process have been identified in model plants such as *A. thaliana* and rice. The most well-known signaling pathway is the SOS (or CIPK/CBL) pathway. In the SOS (salt-overly-sensitive) pathway, the Na^+/H^+ antiporter SOS1 can be phosphorylated by the SOS2/SOS3 complex after the complex is activated by increased cytosolic Ca^{2+} (Hasegawa et al. 2000; Zhu 2002). The *SOS3* gene encodes a member of the calcineurin B-like protein family (CBL) and SOS2 belongs to the CBL-interacting protein kinase family (CIPK). When plants are exposed to salt stress, the increased cytosolic Ca^{2+} binds to SOS3 and then promotes the formation of the SOS2-SOS3 complex. The binding of SOS3 releases the catalytic domain of SOS2 that is then able to phosphorylate SOS1 (Qiu et al. 2002; Zhu 2002). The SOS pathway was characterized in *Arabidopsis* but is conserved in rice. In addition to the cloning of *OsSOS1*, the *OsSOS2*/*OsCIPK24* and *OsSOS3*/*OsCBL4* genes were also cloned from rice based on sequence similarity, and their functions and relationships were studied in yeast and *A. thaliana* (Martinez-Atienza et al. 2007). The results showed that the three rice proteins are able to functionally replace their homologs in *A. thaliana*, indicating that the SOS pathway is conserved between monocots and dicots.

In addition to the CIPK/CBL pathway, the CDPK (calcium-dependent protein kinase) proteins were also found to be essential for rice salt tolerance. Either of the CDPK genes *OsCPK21* or *OsCPK12* can enhance salt tolerance in rice. Overexpression of *OsCPK21* upregulated ABA- and salt stress-inducible genes, while overexpression of *OsCPK12* was shown to upregulate genes encoding ROS scavenging enzymes such as OsAPx2 and OsAPx8 (Asano et al. 2011, 2012). Several other genes that encode kinases were also studied for their roles in salt tolerance, including the receptor-like protein kinase gene *OsRPK1* and the phosphoglycerate kinase gene *OsPGK2*. Overexpression of these genes in *A. thaliana* or tobacco showed that they also contribute to salt tolerance in plants (Joshi et al. 2016; Shi et al. 2014).

There are other salt stress signaling pathways that have been identified in *A. thaliana*, such as the MAPK and phospholipid pathways (Zhu 2002), but their existence in rice requires further investigation. However, several studies have suggested that there are many signaling components that regulate salt tolerance in rice. The rice phospholipase Dα was found to be important in the activation of H^+-ATPase and salt tolerance (Shen et al. 2011), and a phospholipase C, OsPLC1, can elicit stress-induced Ca^{2+} signals and controls Na^+ accumulation in the leaf (Li et al. 2017), supporting the idea that the phospholipid pathway is also important for rice

salt tolerance. Additionally, a rice calmodulin-like gene, *OsMSR2*, and a G-protein-encoding gene, *OsYchF1*, were also suggested to be involved in signaling in the rice salt stress response (Cheung et al. 2013; Xu et al. 2011).

16.8 Perspective

In the past several decades, researchers have identified numerous genes involved in salt tolerance in rice, and several of them have been used to engineer salt-tolerant rice varieties. However, most of this progress was achieved through reverse genetics, and the basic knowledge was acquired from studies conducted in *A. thaliana*. Because rice is a monocot crop and *A. thaliana* is a dicot weed, differences in the molecular mechanisms underlying salt tolerance between these two diverse species could very well be extensive. We therefore postulate that a large number of salt tolerance-related genes remain to be identified in rice. However, our current knowledge of the genes and pathways that regulate salt tolerance is still very limited, and a clear picture of the gene networks involved has yet to emerge. In the future, we believe that more and more related genes will be identified and functionally characterized. Particularly, the development of phenomics and deep-sequencing technology will accelerate this process (Al-Tamimi et al. 2016; Takaqi et al. 2015). These progresses will not only greatly improve our understanding of the molecular and genetic basis of rice salt tolerance but also contribute to fast breeding of salt-tolerant rice varieties.

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Chapter 17
Cold and Water Deficit Regulatory Mechanisms in Rice: Optimizing Stress Tolerance Potential by Pathway Integration and Network Engineering

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Abstract The responses of rice to cold and water deficit are multidimensional. A holistic approach to maximize tolerance potential requires the optimization of ideal combinations of multiple interacting entities in a genetic network. This chapter presents a modern view for engineering stress-resilient rice cultivars. The first section summarizes the physiological and biochemical aspects of cold and water deficit at the whole-plant and cellular levels. The second part summarizes the major hubs of signaling and transcriptional regulation that lead to biochemical and physiological changes as validated by functional genomics. The rapidly emerging area of investigation on epigenetic regulatory mechanisms as critical layer of control for fine-tuning is presented in brief in the third section. And finally, the last section summarizes the large-effect QTL for cold tolerance and yield stability under drought. By integrating these four layers of information, this chapter should inspire a holistic approach for stress tolerance engineering with strategies illuminated by systems-level biology.

Keywords Abscisic acid · Antagonism · Calcium signaling · Cellular dehydration · Genetic background · Genetic networks · Molecular synergy · QTL · Reactive oxygen species · Regulatory hubs · Transcription factors

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17.1 Introduction

Functional genomics elucidated how individual genes or proteins function in context of cellular physiology and biochemistry. While this has led to transformative advances, it is important to reiterate that the mechanisms by which the cell responds to growth and environmental signals are much more complex than the function of just one regulatory, biochemical, or signaling protein. The physiological basis of cold and water deficit stresses is multidimensional with cascading *domino effects*. Under such complexity, earlier efforts to identify the master gene(s) for tolerance by implicating one or few regulatory switch(es) or biochemical step(s) led to only modest tangible outcomes in cultivar enhancement, contrary to what was expected from the outcomes of foundational functional genomics studies. One type of defense often translates to enhanced plant survival but not necessarily reduced penalty to yield.

As the paradigm continues to evolve, the reductionist point of view based on the master switch concept is now being critically reevaluated for its true agronomic potential, as it appears to be an oversimplification of the true nature of intricately interacting cellular processes. While genetic interactions are crucial to the expression of stress tolerance potential, unintended effects of such interactions often cause major biological trade-offs that dampen the expected positive outcome. There is a growing appreciation that engineering for stress tolerant rice would require pyramiding optimal combinations of positively interacting network hubs to create synergistic, complementary, and non-antagonistic effects, tailored to specific genetic background and growth environment.

This chapter presents all aspects of cellular and genetic mechanisms for cold and water-deficit stresses and highlights opportunities for synergistic effects in rice. The intention is to inspire critical thinking away from the master switch paradigm. Despite recent advances in molecular genetics, a large gap still exists between the progress in understanding regulation through gene-by-gene functional studies and the need to elucidate the regulatory aspects of QTL effects. This chapter integrates all pertinent layers of molecular control in parallel to QTL discovery to inspire systems-level approaches for stress tolerance breeding in rice.

17.2 Confounding Effects of Stress in Rice

17.2.1 Cold

Chilling has the most severe impacts during the early stages of seedling establishment and during reproductive development. In general, the more tolerant genotypes tend to be japonicas, many of which tolerate temperatures around 13 °C (Redona and MacKill 1996). Seedling emergence and establishment could be severely impaired even with periodic cold snaps (Morsy et al. 2005;

Suzuki et al. 2008). Continuous cold for up to few weeks during seedling establishment lead to rapid leaf chlorosis, premature senescence, and eventually seedling death (Andaya and Mackill 2003a, b).

Oxidative stress is an early event that triggers a cascade of perturbations during exposure to damaging cold (Cheng et al. 2007; Yun et al. 2010). Its negative effects progressively become more complex and irreversible with time, especially in sensitive genotypes lacking the capacity for timely defenses against the biochemical malfunctions caused by reactive oxygen species (ROS) (Fig. 17.1, Table 17.1). ROS trigger cellular toxicity through the buildup of toxic by-products of stress metabolism such as malondialdehyde due to lipid peroxidation. They also contribute to the disruption of membrane-bound enzymes and denaturation and aggregation of essential proteins (Lee et al. 2009; Nishida and Murata 1996; Sato et al. 2011). Early defenses require mechanisms for detoxification and repair of macromolecules (Dipiero and Leonardis 1997; Park et al. 2010). Membrane function is often compromised by altered viscosity of lipid bilayer, reduced cytoplasmic streaming, or solute concentration effects (Fig. 17.1, Table 17.1) (Beck et al. 2007; Freitas et al. 2016). Results are membrane blebs and aggregation and consequently leakage of cytoplasmic components (Los and Murata 2004). Physical changes in membrane and reduced cytoplasmic streaming impair the activation energy of membrane-bound enzymes, which further promote ROS accumulation (Lyons 1973; Nishida and Murata 1996). Cold stress also disrupts chloroplast development and inhibits photosynthesis by altered oxidation of PSII, disrupted thylakoid electron transport, and impaired carbon reduction cycle (Fig. 17.1, Table 17.1) (Allen and Ort 2001; Huner et al. 1993; Rapacz et al. 2004; Xiaochuang et al. 2017; Zhang et al. 2014).

Sensitive rice genotypes are particularly most vulnerable to cold during the early stages of microspore development and during anthesis, causing pollen and spikelet sterility (Farrell et al. 2006; Jagadish et al. 2010; Perreira da Cruz et al. 2006; Satake 1991). Cold-induced spikelet sterility is caused by the inhibition of pollen tube elongation (Shinada et al. 2013; Matsui and Kagata 2003). Compromised ROS scavenging systems contribute to drastic alterations of cellular oxidative balance necessary for normal microspore development. Reduced amount of available sugars due to the impaired photosynthesis is also a major cause (Beck et al. 2007; Paupiere et al. 2014). The former leads to membrane lipid peroxidation and irreversible damages to reproductive cells, while the latter depletes the source of nourishment for pollen development (Fig. 17.1, Table 17.1).

17.2.2 Water Deficit

The complex nature of drought is a manifestation of its confounding effects especially in combination with heat. Rice is very sensitive to drought during early seedling growth, but yield is most severely affected when it occurs at booting stage until grain filling, during which photosynthates are being partitioned between

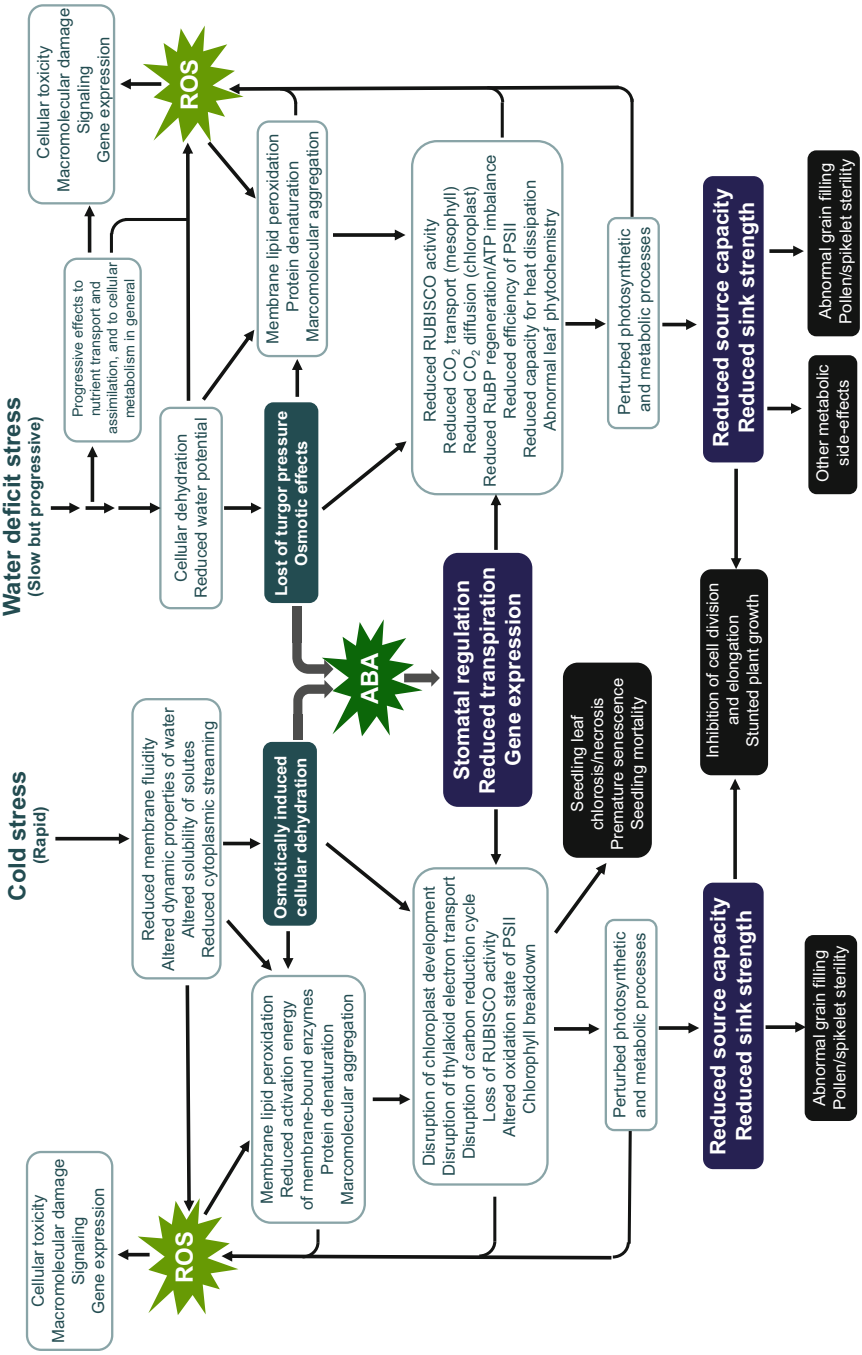
Cold stress
(Rapid)
Water deficit stress
(Slow but progressive)
Reduced membrane fluidity
Altered dynamic properties of water
Altered solubility of solutes
Reduced cytoplasmic streaming
Osmotically induced cellular dehydration
Cellular dehydration
Reduced water potential
Progressive effects to nutrient transport and assimilation, and to cellular metabolism in general
Lost of turgor pressure
Osmotic effects
ABA
Stomatal regulation
Reduced transpiration
Gene expression
Membrane lipid peroxidation
Reduced activation energy of membrane-bound enzymes
Protein denaturation
Marcomolecular aggregation
Membrane lipid peroxidation
Protein denaturation
Marcomolecular aggregation
Disruption of chloroplast development
Disruption of thylakoid electron transport
Disruption of carbon reduction cycle
Loss of RUBISCO activity
Altered oxidation state of PSII
Chlorophyll breakdown
Reduced RUBISCO activity
Reduced CO_2 transport (mesophyll)
Reduced CO_2 diffusion (chloroplast)
Reduced RuBP regeneration/ATP imbalance
Reduced efficiency of PSII
Reduced capacity for heat dissipation
Abnormal leaf phytochemistry
Perturbed photosynthetic and metabolic processes
Perturbed photosynthetic and metabolic processes
Seedling leaf chlorosis/necrosis
Premature senescence
Seedling mortality
Inhibition of cell division and elongation
Stunted plant growth
Reduced source capacity
Reduced sink strength
Reduced source capacity
Reduced sink strength
Other metabolic side-effects
Abnormal grain filling
Pollen/spikelet sterility
Abnormal grain filling
Pollen/spikelet sterility
ROS
ROS
Cellular toxicity
Macromolecular damage
Signaling
Gene expression
Cellular toxicity
Macromolecular damage
Signaling
Gene expression

Fig. 17.1 Network of physiological perturbation triggered by cold or water deficit in rice. The initial events, i.e., *cellular dehydration by physical or osmotically induced effects*, are defined by overlapping properties that lead to *domino effects*. Negative consequences of these perturbations are manifested during the most sensitive stages of plant development particularly during early vegetative growth and during reproduction development and embryo maturation. The more profound effects of water deficit are attributed to its slow but progressive nature, which cause lingering periods of perturbed cellular status and hence more complex metabolic side effects

Table 17.1 Biochemical dysfunctions and physiological perturbations caused by cold (**C**) and water-deficit (**D**) stresses at the cellular and whole-plant levels. Induced defenses and constitutive avoidance properties that address each dysfunction/perturbation for stress tolerance enhancement are listed, with examples in parenthesis whenever known

Injury/cellular dysfunctions	Inducible defenses*	Avoidance properties
Osmotic effect or solute concentration effect	***C/D:*** Osmotic adjustment (*proline, glycine betaine, polyamines, soluble sugars, K^+*)	
Cellular dehydration	***C/D:*** Cell and tissue water facilitation (*water channel proteins*, *aquaporins*)	***D:*** Efficient water uptake *(enhanced root growth, deep and thick root system, root hair proliferation)*
		D: Water use efficiency (*leaf morphology and photochemistry, mesophyll cell, and stomatal architecture*)
		C/D: Glaucousness of leaves and tiller sheaths (*epicuticular* waxes)
Membrane destabilization	***C:*** Membrane-protective proteins (*LTI6A, LTI6B, dehydrins, LEA proteins*)	–
	C/D: Membrane lipid remodeling (*fatty acid desaturases, FAD*)	–
	D: Protection against membrane lipid peroxidation (*vitamin E, α-tocopherol*)	–
Protein denaturation and aggregation	***C/D:*** Chaperones (*α-crystallin heat shock proteins*, *dehydrins, LEA proteins*)	–
Oxidative injuries	***C/D:*** Radical scavenging (*superoxide dismutases, catalase, peroxidase, glutathione-S-transferase, ascorbate peroxidase*)	
Cellular toxicity	***C/D:*** Detoxification and efflux (*multidrug resistance proteins, MRPs; multidrug and toxic compound extrusion, MATE, proteins)*	–
Inhibition of cell division and elongation; stunted growth; impaired/abnormal reproductive growth	***C/D:*** Turgor maintenance, cell cycle timing regulation (*unknown*)	***C/D:*** Early flowering, floral organ development synchronized with drastic fluctuations in temperature and moisture conditions (*unknown*)

(continued)

Table 17.1 (continued)

Injury/cellular dysfunctions	Inducible defenses*	Avoidance properties
		C: Enhanced stress germination potential and seedling vigor, timely and robust emergence (*unknown*)
		C/D: Protective morphological attributes of floral organs (*unknown*)
Impaired photosynthesis and assimilate partitioning	***C/D:*** Stable RUBISCO, efficient CO2 diffusion and transport, mesophyll cell architecture, better isoforms of biosynthetic enzymes *(unknown)*	***D:*** Protection of photosynthetic apparatus, enhanced stem reserve mobilization potential, sink strength, accelerated grain filling, regulation of senescence (*unknown*)

This table must be interpreted within the context of the network of biochemical and physiological injuries presented in Fig. 17.1
*C = cold; D = Dehydration

source and sink organs (Henry et al. 2015; Manikavelu et al. 2006; Pieters and Souki 2005). Progressive reduction in leaf water potential during the early progression of drought creates a state of cellular dehydration (Fig. 17.1, Table 17.1). This physiological state is characterized by a decline in cell turgor, which affects many aspects of cell growth by inhibiting cell division and elongation (Chaves et al. 2009; Farooq et al. 2009; Serraj and Sinclair 2002). Root-shoot signaling by abscisic acid (ABA), H_2O_2, and Ca^{2+} triggers a response in the leaves by regulating stomatal aperture to prevent rapid transpiration (Chaves et al. 2009; Knight et al. 1996). Therefore, the origins of drought-induced perturbations are biochemical events triggered by cellular dehydration and its accompanying stomatal closure, which lead to direct and/or indirect effects (Fig. 17.1). If drought progressed over a longer period, photosynthesis, cell division, and cell elongation are progressively impaired, stunting plant growth (Farooq et al. 2009; Reddy et al. 2004).

Direct cellular effects of physiological water deficit involve osmotic stress (Serraj and Sinclair 2002). Similar to a situation when extracellular water gradually freezes, water moves out of the cytoplasm to balance the osmoticum. As cytoplasmic hydration status declines, osmotically induced dehydration effects lead to irreversible injuries through protein/enzyme denaturation and aggregation and disruption of other macromolecules including lipids and membranes (Berlett and Stadtman 1997). These events affect virtually every aspect of cellular metabolism, especially the processes required for cell division and elongation. Stunted growth is due to the loss of cellular components that drive biochemical reactions and transport. Damage to critical proteins also leads to biochemical malfunctions that trigger toxicity by ROS (Antoine et al. 2005; Baxter et al. 2014; Gigon et al. 2004; Munne-Bosche et al. 2005; Pyngrope et al. 2013; Sharma and Dubey 2005).

Indirect effects of physiological water deficit are primarily due to the impacts on metabolism and photosynthesis (Fig. 17.1). While the responses of the leaves through stomatal closure are important mechanisms for modulating transpiration, the same process triggers dysfunctions because of drastic decline in Rubisco activity (Lawlor 2002; Parry et al. 2002; Vu et al. 1999). Stomatal closure is an impediment to the process of CO_2 transport by mesophyll cells and diffusion into the chloroplast. The consequences are impaired RuBP regeneration, reduced CO_2 metabolic capacity, impaired photosystem II, and reduced capacity for heat dissipation (Chaves et al. 2009; Cornic and Massacci 1996; Li et al. 2012; Pieters and Souki 2005; Reddy et al. 2004). These perturbations not only lead to elevated ROS, but most importantly, they are the main reasons for the long-term negative impacts of drought by affecting the efficiency by which photosynthates are produced and partitioned between defense and growth-related processes (Amahdi and Baker 2001; Yang et al. 2001; Zhou et al. 2007). Impaired photosynthesis compromises the biochemical processes that fuel cell division and elongation in support of vegetative growth and ultimately reproductive growth (Fig. 17.1). As physiological disturbance intensifies with increasing severity of water deficit, impaired photosynthesis limits source capacity, reducing assimilate translocation to the sink organs during microspore development and grain filling (Asch et al. 2005; Li et al. 2012; Liu and Li 2005).

17.2.3 Crossroads of Cold and Water-Deficit Response Mechanisms

Progressive water deficit gradually creates a state of cellular dehydration and loss of cell turgor (Fig. 17.1). Similarly, injurious levels of chilling impair cytoplasmic streaming, which mimic cellular dehydration effects by osmotically induced solute concentration (Bray 2002). When the temperature drop is steep and rapid, cells experience an osmotically induced dehydration, manifested by the typical symptoms of leaf wilting and stomatal closure (Choi et al. 2000). Low soil and water temperatures promote ABA synthesis similar to gradual root drying during drought (Chaves et al. 2009; Freitas et al. 2016; Park and Baek 2012).

Cellular dehydration caused by water deficit or cold affects membrane function. Cold alters the dynamic movement of water molecules, which in turn affects solute solubility and promotes membrane rigidity (Lyons 1973; Nishida and Murata 1996). Biochemical malfunctions induced by water deficit and cold trigger the buildup of ROS beyond what could be handled by scavenging mechanisms. Membrane lipid peroxidation triggered by water deficit and cold further aggravates the physical injuries to the membrane, which subsequently impair cell division and elongation. Cellular dehydration contributes to the loss of macromolecular integrity, which perturbs photosynthesis, metabolism, and energy balance (Beck et al. 2007). Ultimately, both stresses negatively affect photosynthesis by limiting CO_2

absorption and transport and other side effects to the photosynthetic machinery (Fig. 17.1).

Beyond the similar properties, there are distinct contexts to cold and water deficit. The impacts of cold are often abrupt and can happen overnight, while the impacts of water deficit are progressive. The effects of cold mimic many but certainly not all of the cellular strains imposed by gradual but persistent water deficit. Transport and assimilation of nutrients are important processes that are negatively affected when water is severely limiting. Impairment in transport and assimilation is relatively milder when the plant is subjected to cold but water is not limiting (Fig. 17.1). Drought is also a combined effect of water deficit and heat. Therefore, its physiological effects are more encompassing because of secondary and tertiary strains that carry over through the later stages of development due to limited photosynthesis and source-sink allocation (Allen and Ort 2001).

Defense mechanisms addressing various biochemical dysfunctions are quite well investigated in rice (Table 17.1, Fig. 17.1). These mechanisms include osmotic adjustment, cell and tissue water facilitation, cell membrane remodeling and stabilization, fatty acid desaturation, repair and stabilization of proteins, and enhanced radical scavenging (Antoine and Stewart 2005; Apel and Hirt 2004; Desikan et al. 2001; Miquel et al. 1993; Morsy et al. 2005; Murata et al. 1992; Serraj and Sinclair 2002; Steponkus et al. 1998; Yu et al. 2006). Enhancing the activities of any one of these mechanisms has proven inadequate to maximize stress tolerance potential especially in context of yield. Enhancement in stress tolerance in terms of reduced injuries and better plant survival has been achieved by genetic engineering strategies. Beyond such enhancements, reducing the penalty to net growth and grain yield is obviously much more complex than engineering for a single biochemical defense (Table 17.1).

17.3 Cold and Water-Deficit Signal Transduction and Transcriptional Networks

Responses to cold and dehydration are initiated by the perception of chemical signals, most importantly ABA and ROS. ABA level is regulated by the rate of its synthesis, conjugation, and degradation (Cutler et al. 2010). ROS homeostasis is achieved by balancing production from metabolic processes and physiological perturbation with scavenging mechanisms (Foyer and Noctor 2005; Neill et al. 2002; Wang et al. 2016). This process ensures that ROS production is kept under check through a feedback loop that constantly alarms the scavenging pathways and their associated signaling mechanisms.

Signal perception is amplified by second messengers that trigger a cascade of protein modification. Ca^{2+}, inositol triphosphate (IP_3), cyclic AMP/GMP (cAMP, cGMP), diacylglycerol (DAG), phosphatidic acid (PA), and nitric oxide (NO) function as second messengers that trigger enzyme activation/inactivation,

intracellular ion transport, and activation of transcriptional regulatory proteins. Components of these mechanisms are evolutionarily conserved in flowering plants and have been validated by functional genomics in rice.

17.3.1 Signal Perception Through ABA and ROS and Amplification by Ca^{2+}

Understanding the mechanisms by which cold and water deficit are perceived through ROS and ABA is critical for elucidating how the upstream components of signaling interpret the signals to the nucleus or cytoplasmic components (Fig. 17.2). These mechanisms were illuminated by the receptors for ABA and

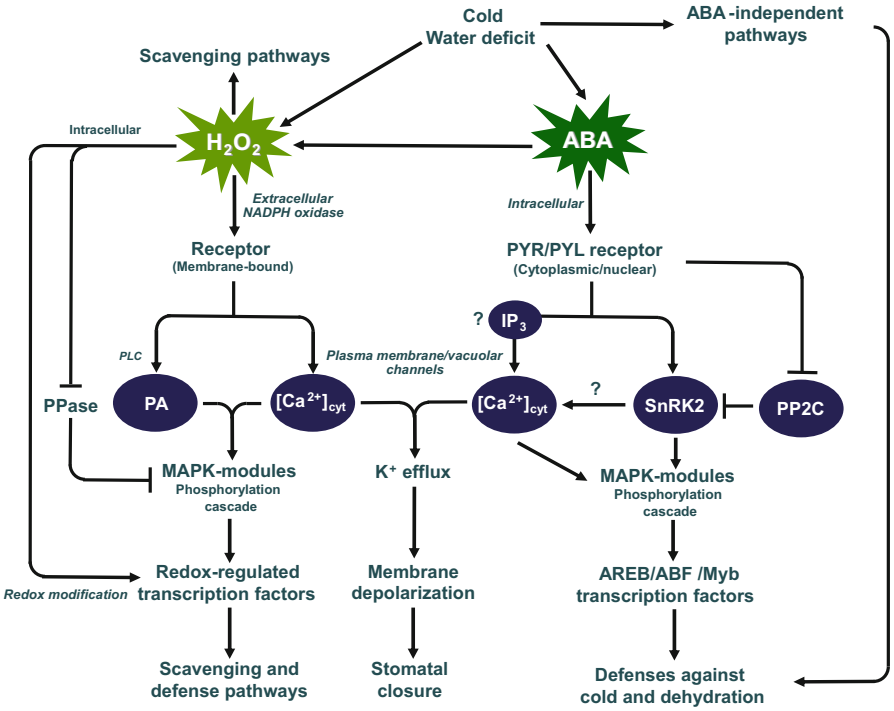

Fig. 17.2 Signaling pathways involved in the perception and transduction of H_2O_2 and ABA during cold and water deficit. ABA is perceived by the PYR/PYL class of cytosolic or nuclear proteins. Interaction between ABA and H_2O_2 feeds to a common second messenger Ca^{2+}. The $[Ca^{2+}]_{cyt}$ signatures facilitate rapid physiological changes by inducing membrane depolarization, which triggers rapid stomatal closure, and indirectly facilitates biochemical and physiological changes through the activation of specific classes of regulatory transcription factors. The process is conserved between *Arabidopsis* and rice. *PPase* Protein phosphatase, *PLC* phospholipase-C, *PP2C* protein phosphatase 2c, *SnRK2* SNF1 protein kinase 2, *IP_3* inositol triphosphate

ROS. Hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2^{\cdot}$), superoxide anion (O_2^{-}), and hydroxyl radical (OH) are produced in membranes, chloroplasts, and mitochondria (Bode et al. 2016; Chu et al. 2010; Juszczak et al. 2016; Kim et al. 2012; Buer et al. 2016). Cells are protected through ROS scavenging by peroxidase, superoxide dismutase, catalase, thioredoxin, glutathione reductase, tocopherol, and glutathione. Extracellular ROS especially H_2O_2 produced by NADPH oxidases acts as rapid and systemic signal (Distelbarth et al. 2013; Kim et al. 2012).

The precise mechanism by which H_2O_2 is perceived is not fully understood. However, the emerging theme is that extracellular H_2O_2 is perceived by an unknown ROS receptor similar to histidine kinases of bacterial two-component systems that can sense both extracellular and intracellular H_2O_2 (Apel and Hirt 2004; Baxter et al. 2014). Perception of H_2O_2 by the redox receptor generates PA through phospholipase-D and triggers the release of Ca^{2+} from the cell wall and vacuole. These events activate the mitogen-activated protein kinase (MAPK) cascade, leading to either activation or repression of transcription factors. Additionally, intracellular H_2O_2 could directly regulate certain components of MAPK cascade by repressing dephosphorylation. Intracellular H_2O_2 could also directly activate certain redox-sensitive transcription factors (Fig. 17.2).

ABA is perceived by the PYR/PYL/RCAR family of cytosolic or nuclear proteins, encoded by more than ten genes in both *Arabidopsis* and rice (Ma et al. 2009; Park et al. 2009; Tian et al. 2015). The PYR/PYL-mediated transduction of ABA occurs through a signaling complex (Chen et al. 2012; Park et al. 2009). One component of this complex is the SNF1-related protein kinase SnRK2, which is involved in regulating stomatal closure through its direct control of K^+/Cl^- efflux (Figs. 17.2 and 17.3). SnRK2 kinases are encoded by a small gene family with about ten members in both *Arabidopsis* (SnRK2/1) and rice (OsRK1). The second component is the type-2c protein phosphatase PP2C, which represses SnRK2 by dephosphorylation. In the absence of ABA, the repressed SnRK2 is unable to phosphorylate the AREB/ABF transcription factors; thus the repressed state of SnRK2 shuts off ABA-regulated transcriptional network. ABA binding to the PYR/PYL receptor causes its conformational change that encapsulates ABA, and exposes a hydrophobic surface that interacts with PP2C, causing inhibition of its phosphatase activity (Park et al. 2009). PP2C inhibition releases SnRK2 from repression, hence activation of AREB/ABF transcription factors (Figs. 17.2 and 17.3).

It is known that Ca^{2+} is directly involved in the initiation of H_2O_2-mediated MAPK phosphorylation cascade. Undoubtedly, Ca^{2+} plays a central role in the amplification of the perceived cold and water-deficit signals either through ABA, ROS, or both (Fig. 17.2). Seminal studies in *Arabidopsis* have shown that transient $[Ca^{2+}]_{cyt}$ is required for gene expression. In addition to H_2O_2, other second messengers including IP_3 also contribute to the regulation of $[Ca^{2+}]_{cyt}$ dynamics (Kiegle et al. 2000; Knight and Knight 2000; Knight et al. 1996; Polisensky and Braam 1996). The specificity of downstream responses involves the establishment of distinct $[Ca^{2+}]_{cyt}$ signatures dictated by the combinatorial effects of different stimuli (Dodd et al. 2006; Hirschi 1999; Kiegle et al. 2000; Marti et al. 2013). Signals originating from $[Ca^{2+}]_{cyt}$ are transduced by Ca^{2+} sensors, including

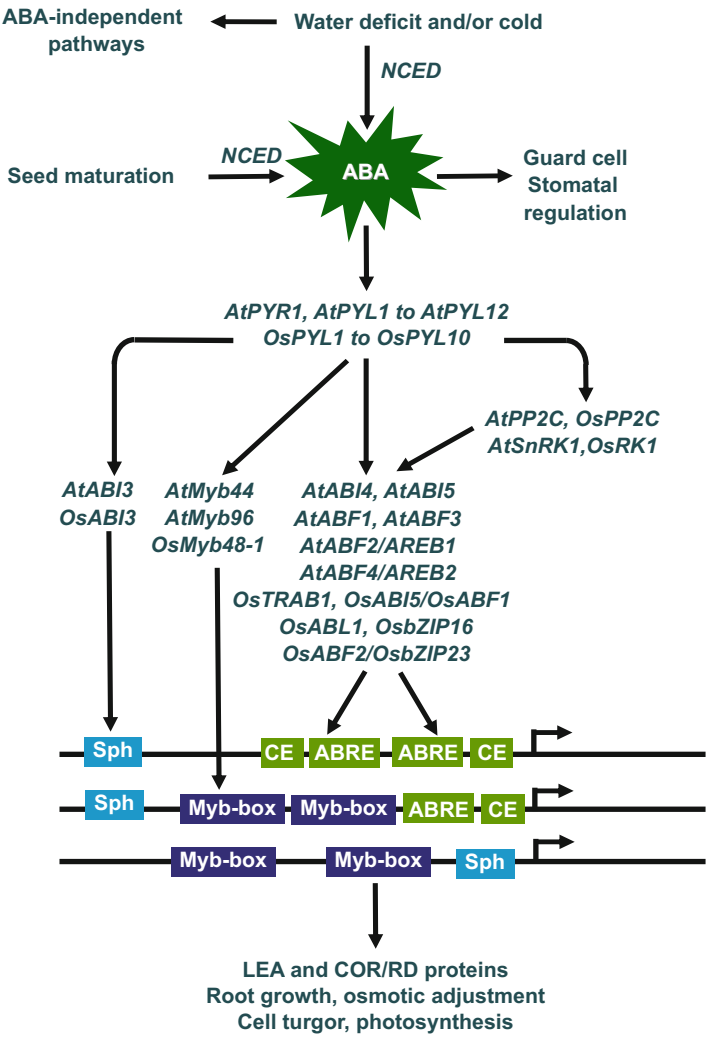

Fig. 17.3 ABA-dependent transcriptional network that is highly conserved between *Arabidopsis* and rice. Cold and water deficit induce the biosynthesis of ABA through the 9-*cis*-epoxycarotenoid dioxygenase (NCED) pathway. Apart from causing membrane depolarization in guard cells and rapid stomatal closure, perception of ABA activates the ABF/AREB (ABA factors/ABA-response element binding) transcription factors, as well as certain ABI3/VP1 and Myb transcription factors. Some transcription factors are activated by phosphorylation by the ABA-receptor complex of SnRK1/2 kinase (*Arabidopsis*) or OsRK1 (rice) and protein phosphatase AtPP2C/OsPP2C, upon binding of ABA to AtPYR/PYL/OsPYL. Activation of ABA-responsive genes is facilitated by a number of cis-elements, of which the most dominant are the ABA-response complex (ABRC) comprised of *ABRE* and coupling factors *CE*. Other minor classes of ABA-response enhancers include *Myb-box*-like and *Sph* elements. ABA-mediated transcriptional network activates genes involved in protecting macromolecules against denaturation and aggregation and regulation of root growth and photosynthesis

calmodulin (CaM), calcineurin-B like (CBL), calmodulin-binding kinases (CIPK), and Ca^{2+}/calmodulin-domain protein kinases (CDPK). These sensors are highly conserved between *Arabidopsis* and rice (Cheong et al. 2003; Huang et al. 2011; Kim et al. 2003; Kolukisaoglu et al. 2004).

17.3.2 ABA-Dependent Transcriptional Networks

The final outcome of ABA perception is either a short-term or long-term response to cold or dehydration. Short-term response is primarily physiological in nature. It facilitates rapid stomatal closure through $[Ca^{2+}]_{cyt}$-mediated regulation of K^+ and Cl^- efflux, triggering membrane depolarization. Genes involved in this short-term response have already been characterized in both *Arabidopsis* and rice (Fig. 17.3). While also mediated by Ca^{2+} signaling, the long-term response configures physiological changes by coordinated activation or repression of a battery of stress-related genes involved in cellular adjustment or defense mechanisms (Santiago et al. 2009). In rice, cold- and dehydration-regulated expressions of at least 1000 genes are known as downstream effects of ABA signaling, although many of these genes may also be regulated independent of ABA (De los Reyes et al. 2015; Lenka et al. 2009; Narusaka et al. 2003). The major hubs of these transcriptional networks are specific members of ABI3VP1, bZIP, and Myb transcription factor families that interface the upstream ABA signaling relay with the transcriptional machinery (Fujita et al. 2013; Yoshida et al. 2015). Members of ABI3VP1 are involved primarily in gene expression during embryo maturation and dormancy through the *Sph* cis-elements (Suzuki et al. 1997).

The central roles of bZIP transcription factors in configuring ABA-mediated transcriptional networks are well established (Fig. 17.3). Among the most characterized is the ABRE-binding proteins/ABRE-binding factors (AREB/ABF), which regulate their downstream target genes by binding to the ABA-responsive element (ABRE), G-box, or C-box with the consensus sequence of CGMC**ACGT**B and characteristic "ACGT" core motif (De los Reyes et al. 2015; Guiltinan et al. 1990; Ma et al. 2009; Nakashima and Yamaguchi-Shinozaki 2013; Yun et al. 2010). ABRE functions in combinatorial manner requiring multiple copies and other non-ACGT-type coupling elements (CE1, CE3), forming the ABA-response complex or ABRC (Hobo et al. 1999; Narusaka et al. 2003; Shen and Ho 1995; Shen et al. 1996). Binding of AREB/ABFs to ABRC is regulated through phosphorylation by SnRK2 (Furihata et al. 2006; Nakashima and Yamaguchi-Shinozaki 2013). ABA-dependent gene expression involves *AtAREB1*/*AtABF2*, which is conserved in rice, i.e., *OsABF2* (Fig. 17.3) (ZG et al. 2014; Li et al. 2013). Two other members of the AREB/ABF subfamily (*AtABF3*, *AtABF4*) function synergistically with *AtAREB1*/*AtABF2* for the establishment of full ABA transcriptional network (Choi et al. 2000; Yoshida et al. 2010). Another bZIP protein *AtABI5* is a key player in the regulation of ABA-dependent gene expression primarily during embryo maturation (Finkelstein and Lynch 2000; Lopez-Molina et al. 2001; Shen

et al. 1996). The ortholog of *AtABI5* in rice (*OsABI5*) performs very similar functions (Zou et al. 2008).

OsTRAB1 (*transcription factor responsible for ABA regulation-1*) is a major regulator of ABA-dependent transcriptional network in rice by binding to the ABRC (Fig. 17.3) (De los Reyes et al. 2015; Hobo et al. 1999; Xue and Loveridge 2004). Like the *Arabidopsis* AREB/ABFs, *OsTRAB1* and other similar factors such as *OsOREB1* and *OsABP9* are activated by phosphorylation by *OsRK1*, which is orthologous to *AtSnRK2* (Chae et al. 2007; Kagaya et al. 2002; Kobayashi et al. 2005). In addition to *OsTRAB1*, other members of the subgroup-A bZIP are important for ABA-dependent drought response transcriptional networks in rice, including several positive regulators such as *OsbZIP12*/*OsABF1*, *OsbZIP71*/*72*, and *OsbZIP66* (Chen et al. 2012; Hossain et al. 2010; Liu et al. 2014; Zou et al. 2008). *OsbZIP23* regulate metabolic adjustments and ABA biosynthesis (Xiang et al. 2008).

The R2R3 subgroup of Myb transcription factors represents another major class of regulatory proteins with central roles in ABA-dependent transcriptional networks (Fig. 17.3). These transcription factors regulate their target genes by binding to the *Myb-box*-like cis-elements (Xiang et al. 2008; Yoo et al. 2005). In *Arabidopsis*, the most prominent ABA-dependent Myb transcription factors for drought are *AtMyb60*, *AtMyb44*, *AtMyb48-1*, *AtMyb2*, and *AtMyb15*, which are involved in a broad range of stress physiological processes including ABA biosynthesis, stomatal regulation, and metabolic adjustments (Abe et al. 2003; Jaradat et al. 2013; Xiong et al. 2014). Similarly, *OsMyb2* and *OsMyb4* are major regulators of cold and dehydration response transcriptomes in rice (Park et al. 2010).

17.3.3 ROS-Facilitated Transcriptional Networks

H_2O_2 is produced by a number of mechanisms including the plasma membrane-bound NADPH oxidases (Foyer and Noctor 2005; Miller et al. 2010; Wang et al. 2016). ABA also causes rapid increase in cellular H_2O_2 via phospholipase-D to establish $[Ca^{2+}]_{cyt}$ that trigger physiological responses and gene expression (Apel and Hirt 2004; Baxter et al. 2014; Cheng et al. 2007; Neill et al. 2002; Park et al. 2010). Current hypotheses suggest that the most direct links between H_2O_2 perception and transcription are through constitutively expressed, redox-sensitive transcription factors or redox-regulated protein kinases (Fig. 17.2). The output of this process is an oxidative-regulated transcriptome, involving a subset of defense-related genes with common denominator cis-elements. Seminal studies in *Arabidopsis* revealed that *as1*/*ocs*/*TGA*-like cis-elements with the consensus "AATTTGAT" and "TAATTTGA" and TGA core motif are primarily responsible for oxidative-regulated expression. However, no real consensus has so far been revealed for a master switch (Chen and Singh 1999).

In rice, much of the vegetative injuries during the early stages of cold stress involve oxidative effects. The ability to attenuate this mode of injury has major contributions to differences between indica and japonica rice cultivars. In general, temperate japonica cultivars are able to recover from an episode of cold better than most tropical indica cultivars because of the timely execution of defenses by an oxidative-mediated transcriptional network. This network is comprised of a large number of genes with common features of *as1*/*ocs*/*TGA*-like, *GARE*/*Pyr*-box, and *Myb2*-box elements in their promoters (Fig. 17.4). Peak expression of these genes was coincident with measurable cold-induced spikes in intracellular H_2O_2 and induced by exogenous H_2O_2 (Cheng et al. 2007; Herath 2011; Park et al. 2010; Yun et al. 2010). Transgenic overexpression identified the *OsTGA10*, a member of the oxidative and pathogen defense-associated bZIP transcription factors, and *OsMyb4*, an R2R3-Myb, as critical network hubs. *OsTGA10* represents an early cold-responsive regulator that responds to ROS perhaps through a redox-activated transcription factor (Fig. 17.4). It regulates its target genes including *OsMyb4* through the *as1*/*ocs*/*TGA*-like cis-elements. *OsMyb4* regulates many genes through the *Myb2-box*-like and *GARE*/*Pyr-box*-like cis-elements.

Overexpression studies indicated the upstream position of *OsTGA10* relative to *OsMyb4* and *NFy-β*. Overexpression of both *OsTGA10* and *OsMyb4* did not affect *OsCBF1*/*OsDREB1B* and *OsCBF3*/*OsDREB1A*, further implying that *OsTGA10*/*OsMyb4* network is independent of CBF/DREB (Fig. 17.4). *OsTGA10* and *OsMyb4* enhanced cold, dehydration, and salinity tolerance of transgenic rice at the vegetative stage, because of downstream genes for ROS catabolism, detoxification and efflux, protein structure maintenance by molecular chaperones, and osmotic adjustment. It has been suggested that the *OsTGA10*/*Myb4* network is not as important in *Arabidopsis* as it is in rice, because *Arabidopsis* is inherently tolerant to mild cold that often kills most rice cultivars. *OsTGA10* and *OsMyb4* are located within QTL for cold, dehydration, and salinity tolerance on chromosomes 6 and 4, respectively (Herath 2011; Yun et al. 2010).

17.3.4 ABA-Independent Transcriptional Networks

Cold and dehydration trigger transcriptional changes that configure various processes of cellular adjustment and defense, through a mechanism independent of ABA signaling. The most well-characterized hubs of this network are the CBF/DREB (C-repeat binding factor/dehydration-response element-binding proteins) subclass of AP2/ERF transcription factors that are evolutionary conserved across dicot (*Arabidopsis*) and monocot (rice) plants (Carvallo et al. 2011; Dubouzet et al. 2003; Liu et al. 1998; Stockinger et al. 1997). The *CBF1*/*DREB1B*, *CBF2*/*DREB1C*, and *CBF3*/*DREB1A* regulate their downstream target *COR* (cold-regulated), *RD* (responsive to dehydration), or *LEA* (late embryogenesis abundant protein) genes by binding to their cognate CRT/DRE (C-repeat or dehydration-response element) cis-elements (Fig. 17.5) (Jaglo-Ottosen et al.

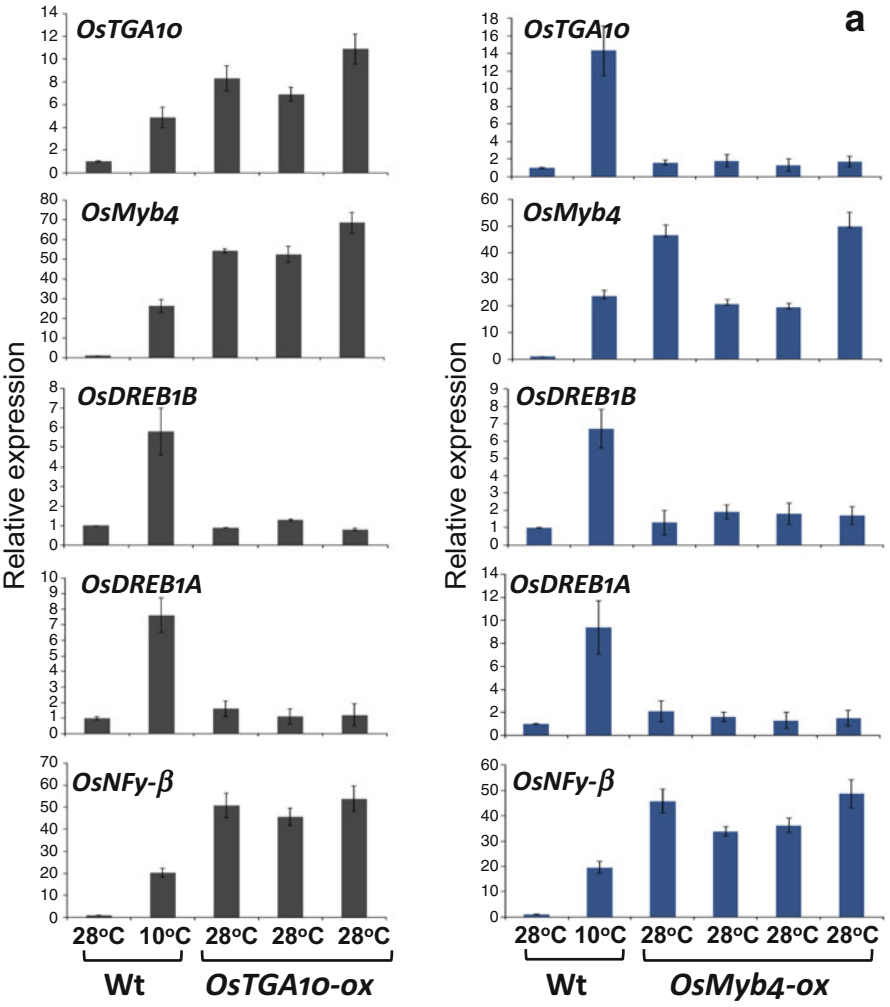

Fig. 17.4 ROS-mediated transcriptional networks associated with the responses of rice to cold, dehydration, or osmotic stress. *OsTGA10* is an upstream regulator responding to cold-mediated spikes in cellular H_2O_2. *OsMyb4* is among the many downstream targets of *OsTGA10*. (**a**) Constitutive expression of *OsTGA10* induces *OsMyb4*, but not vice versa. Independent overexpression of *OsTGA10* and *OsMyb4* does not affect other known transcriptional switches such as Os*DREB1A* and Os*DREB1B*. Constitutive expression of either *OsTGA10* or *OsMyb4* activates another H_2O_2-responsive transcription factor *NFY-β*. (**b**) Targets of *OsTGA10* including *OsMyb4* are activated coordinately in response to cold-induced oxidative signals by binding of *OsTGA10* to the *as1*/*ocs*/*TGA*-like cis-elements. *OsMyb4* binds to either *GARE*/*Pyr-box*-like or *Myb2-box*-like cis-elements. The outcomes of ROS-mediated transcriptional network are the expression of genes involved in radical scavenging, protein stability, osmotic adjustment, efflux mechanisms, source-sink allocation, and growth modulation

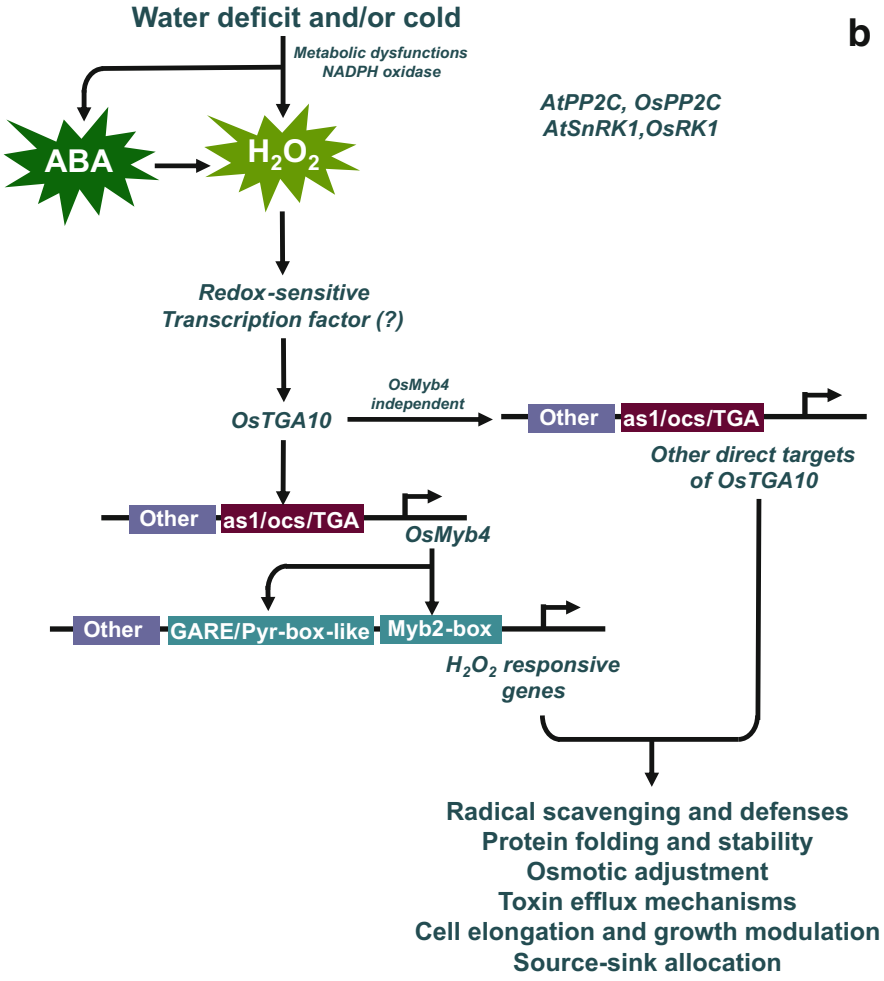

Fig. 17.4 (continued)

1998; Liu et al. 1998; Shinwari et al. 1998). Two other members of the CBF/DREB subclass in *Arabidopsis* (*AtCBF4*, *AtDREB2A*) and one of their orthologs in rice (*OsDREB2A*) have been shown to function specifically for drought-responsive gene expression (Cui et al. 2011; Haake et al. 2002; Sakuma et al. 2006). About 10% of the total genes of rice are regulated through the CBF/DREB regulatory module as evidenced by the distribution of CRT/DRE consensus sequences ((a/g)CCGAC) among a large subset of stress co-regulated genes, and their co-expression in CBF/DREB overexpressing rice (Rabbani et al. 2003; Yazaki et al. 2004).

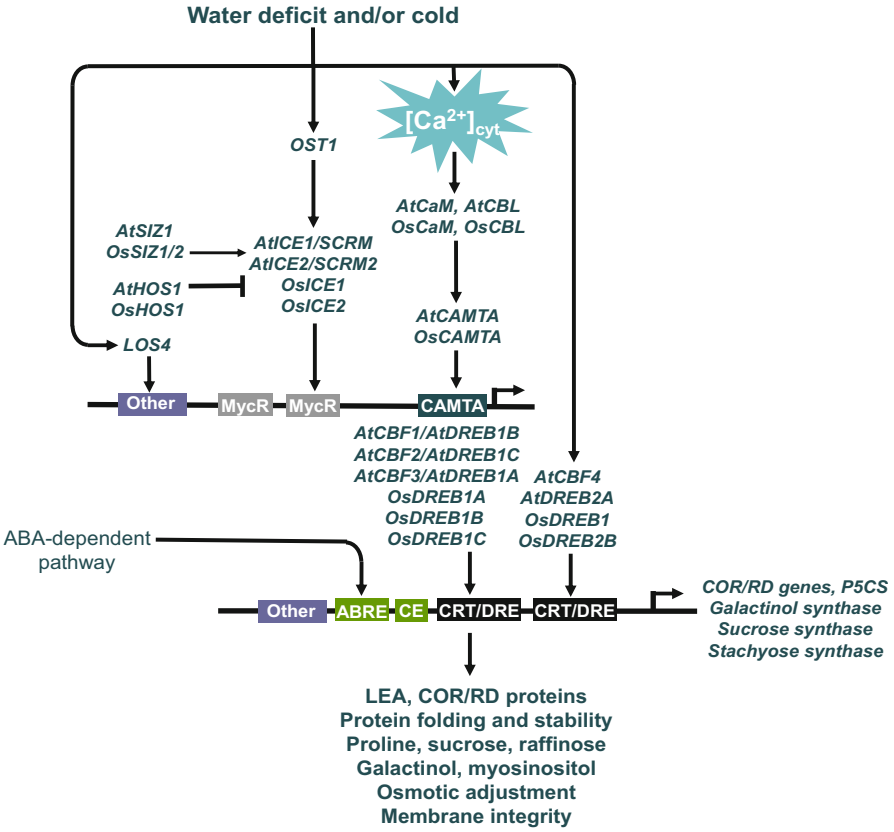

Fig. 17.5 ABA-independent CBF/DREB transcriptional network that is conserved between *Arabidopsis* and rice. $[Ca^{2+}]_{cyt}$ signatures are decoded by specific calcium sensors (AtCaM/OsCaM or AtCBL/OsCBL), which interact with the *AtCAMTA*/*OsCAMTA* transcription factors to derepress the expression of *AtDREB1*/*OsDREB1*. The *AtICE1*/*SCRM* or its rice ortholog *OsICE1* and *AtICE2*/*SCRM2* or its rice ortholog *OsICE2* are activated by cold through *OST1*. The stability of ICE/SCRM proteins is also regulated by AtSIZ1/OsSIZ1/2 or AtHOS1/OsHOS1 and subsequently activates *AtDREB1*/*OsDREB1* through the conserved Myc-like cis-elements in their promoters. Dehydration stress induces the expression of *AtCBF4*/*OsDREB1* or *AtDREB2A*/*OsDREB2B*. Coordinated expression of the downstream targets of the CBF/DREB networks is facilitated by the C-repeat/dehydration-response elements (*CRT*/*DRE*) that are conserved across a battery of LEA and COR/RD genes involved in maintaining protein and membrane stability and in many other genes with known functions in the biosynthesis of compatible solutes

The large number of CBF/DREB regulated genes is consistent with earlier findings that overexpression of *CBF1*/*DREB1B*, *CBF2*/*DREB1C*, and *CBF3*/*DREB1A* enhanced drought and cold tolerance of transgenic plants, mainly as a result of reduced injuries at vegetative stage. Enhancement in tolerance has been attributed to the activation of many genes involved in cellular energetics, carbohydrate metabolism, and biosynthesis of proline, soluble sugars, and sugar alcohols

such as sucrose, raffinose, myoinositol, and galactinol for osmotic adjustment. However, with the large number of genes downstream to CBF/DREB, negative pleiotropic effects have been observed as trade-off. CBF/DREB overexpressors exhibited stunted growth due to misregulation of GA_2-oxidases and accumulation of DELLA repressor proteins (Achard et al. 2008; Cook et al. 2004; Datta et al. 2012; Gilmour et al. 2000; Kasuga et al. 1999; Maruyama et al. 2009; Taji et al. 2002).

The complex regulatory networks configured by CBF/DREB transcription factors are due to multiple layers of control. Each member of CBF/DREB subclass exhibits distinct patterns of spatiotemporal expression, which are consequences of fine-scale upstream regulation in response to cold and/or water deficit. Arabidopsis research elucidated the intricacy of the fine-tuning of CBF/DREB regulon and its downstream networks (Fig. 17.5) (Zarka et al. 2003). For instance, the expression of *CBF3* is regulated at the transcriptional level by the phosphorylated ICE1/SCRM (*inducer of CBF expression-1*/*SCRM*) protein (Chinnusamy et al. 2003; Lee et al. 2005). Under cold conditions, the serine-threonine kinase OST1 (*open stomata-1*) phosphorylates ICE1 to modulate CBF/DREB expression (Ding et al. 2015). Depending on the temperature, the stability and activity of ICE1/SCRM proteins are determined by their ubiquitination and sumoylation by HOS1 and SIZ1 proteins, respectively (Miura et al. 2007). The RNA helicase-like protein LOS4 (*low expression of osmotically responsive genes-4*) positively regulates *CBF3*/*DREB1A* (Gong et al. 2002).

Ca^{2+} is an important second messenger that interfaces the initial cold or dehydration signals to the CBF/DREB regulatory networks. Major players in such pathways are the tonoplast-bound Ca^{2+} channel CAX1 (calcium exchanger-1) and the plasma membrane-bound Ca^{2+}/calmodulin (CaM)-regulated receptor-like kinase CLRK1 (Catala et al. 2003). Both mechanisms contribute to cold and dehydration-associated $[Ca^{2+}]_{cyt}$ signatures. These processes activate the calcineurin-B-like Ca^{2+} sensor CBL1, which then links the calcium-interpreted signals to CBF/DREB transcriptional networks (Fig. 17.5) (Albrecht et al. 2003). In *Arabidopsis*, four functionally redundant CAMTA genes (*CAMTA1*, *CAMTA2*, *CAMTA3*, *CAMTA5*) positively regulate CBF/DREB expression (Doherty et al. 2009; Kim et al. 2013). Pathways leading to the activation of CBF/DREB transcription factors are conserved between *Arabidopsis* and rice (Fig. 17.5). Studies correlating CBF/DREB transcription factors with QTL for cold and/or dehydration across the *Arabidopsis* germplasm revealed their colocation with only few minor QTL (Alonso-Blanco et al. 2005). It has also been established that CBF/DREB regulatory mechanism does not account for the total spectrum of quantitative variation for cold tolerance across the germplasm. Supraoptimal expression of CBF/DREB genes appear to always create major trade-offs to plant fitness (Oakley et al. 2014; Park et al. 2015).

17.4 Potential Contributions of Epigenetic Mechanisms

Epigenetic mechanisms represent another critical dimension of the regulation of developmental and stress-related responses. The regulation of the *Flowering Locus C* (*FLC*) in *Arabidopsis*, which functions as repressor of temperature-regulated flowering, is a classic example of how responses to cold and developmental programs are interfaced through reversible histone acetylation and methylation (Hepworth and Dean 2015). While this developmental mechanism is well established, there is yet a clear example of how regulation at the chromatin level plays a direct role in stress response. Nonetheless, it is well established that developmental and stress responses often intersect; thus the roles of epigenetics in fine-tuning plant responses to cold and dehydration are not hard to envision. Activation of noncoding RNAs (ncRNAs) and transposable elements (TEs) could also contribute to transcriptional network rewiring through posttranscriptional silencing (PTGS) or transcriptional gene silencing (TGS). Indeed, increasing number of studies report the discovery of many ncRNA and retrotransposons that are activated by cold and dehydration in both *Arabidopsis* and rice (He and Amasino 2005; Law and Jacobsen 2010; Shivaprasad et al. 2012) (see also Chaps. 2 and 24).

17.4.1 Regulation by RNA-Directed Posttranscriptional Gene Silencing

RNA-directed PTGS in plants is facilitated by 20–24-nt single-stranded microRNAs (miRNA) generated from hairpin-forming intermediates (Chen 2005). These molecules facilitate cleavage or translational attenuation of their target transcripts, many of which encode transcriptional regulators (Aukerman and Sakai 2003; Kitazumi et al. 2015; Sunkar and Zhu 2004). The miRNA precursors are transcribed by RNA polymerase II and processed by DICER-LIKE 1 (DCL1) and dsRNA-binding protein HYPONASTIC LEAVES 1 (HYL1). The miRNA duplexes are exported to the cytoplasm by HASTY (HST), where they are loaded onto ARGONAUTE (AGO) proteins that guide PTGS (Brodersen and Voinnet 2006; Wu et al. 2010). The miRNA biogenesis is highly conserved between dicot (*Arabidopsis*) and monocot (rice) plants.

PTGS of the ATP sulfurylase gene mediated by *AtmiR395* in response to sulfur starvation represents a seminal demonstration of stress-induced miRNA-mRNA interactions. Subsequently, homologous *OsmiR395* and many other types of miRNAs were discovered in rice. These studies provided unequivocal support to the importance of another layer of control over the stress response transcriptome (Ding et al. 2013). At least 30 different classes of miRNAs related to dehydration stress have already been identified in *Arabidopsis* and rice, many of which have known transcription factor targets (Zhou et al. 2010). One notable example is

AtmiR169g and its rice ortholog *OsmiR169*, which is downregulated by drought in *Arabidopsis* and upregulated in rice. This miRNA is regulated through the CRT/DRE and CBF/DREB transcription factors and targets the *NFy-α* transcription factor (Ni et al. 2013; Zhang et al. 2011; Zhao et al. 2007, 2009). Negative regulation of transcription factor expression by dehydration-induced miRNAs appears to be an important component of regulatory networks. For instance, ABA and dehydration upregulate *miR159*, which modulates the expression of *Myb33* and *Myb101* involved in ABA-mediated networks. The drought-induced *miR396* is involved in the regulation of cell growth through its direct effects on a number of transcription factors. The *miR164* downregulates a NAC-type negative regulator of drought responses (Fang et al. 2014; Kitazumi et al. 2015; Liu et al. 2009; Reyes and Chua 2007; Xia et al. 2012).

Metabolic adjustments during dehydration involve miRNAs as critical fine-tuners. For example, downregulation of *miR171*, *miR397*, and *miR408* that target acid invertases sustained sucrose metabolism during drought, while upregulation of *miR474* suppresses the expression of proline dehydrogenases (Zhou et al. 2010). Regulation of ROS metabolism during dehydration also involves downregulation of *miR528*, which targets peroxidase-encoding transcripts, and *miR398*, *miR397*, *miR408*, and *miR528*, which target the Cu/Zn superoxide dismutase genes *OsCSD1* and *OsCSD2* (Cheah et al. 2015; Lu et al. 2010). There are also known examples of miRNAs involved specifically in cold stress response in rice. The *miR156k*, *miR171a*, *miR319a/b*, *miR168*, *miR1884*, *miR1320*, *miR1876*, and *miR444* are downregulated, while *miR166k/m*, *miR1868*, *miR1435*, and *miR535* are upregulated by cold (Lv et al. 2010). Certain miRNAs facilitate cross-talks between cold and dehydration stresses by virtue of their effects on certain aspects of metabolism. For instance, *miR156* had negative effects on tolerance by virtue of the repression of peroxidase and pyrroline-5-carboxylate synthase (P5CS) (Cui et al. 2015). Manipulation of cold and dehydration transcriptional networks through miRNAs has been attempted in rice. While such attempts led to partial enhancements, most were accompanied by negative pleiotropic effects (Gao et al. 2011; Lin et al. 2012; Yang et al. 2013).

17.4.2 Regulation by DNA Methylation and Transposable Elements

In plants, reversible methylation of cytosine in both symmetric (CHG, CG) and asymmetric (CHH) contexts represents an important mechanism for epigenetic reprograming of the transcriptome. This process has been implicated with responses to cold and dehydration. De novo DNA methylation is induced during cold and dehydration through RNA-directed DNA methylation (RdDM), facilitated by small noncoding RNAs (sRNAs) (Garg et al. 2015; Steward et al. 2002; Wassenegger et al. 1994). Available data support the importance of 24-nt *ra-siRNA*, especially

the *MITE*-derived siRNA in regulating stress-related genes by RdDM (Wassenegger et al. 1994; Wei et al. 2014). Basal methylation of the rice genome has been reported at 44.46% (japonica) and 27.77% (indica) in CG context, 20.14% (japonica) and 13.48% (indica) in CHG context, and 4.02% (japonica) and 3.16% (indica) in CHH context (Li et al. 2012; Sharma et al. 2009). RdDM in rice involves CG methylation in the genic regions and CHC and CHH methylation of TEs (Pélissier et al. 1999). Methylation at promoters is negatively correlated with transcription, while methylation in gene bodies is positively correlated with transcription and overlaps with siRNA loci (Law and Jacobsen 2010; Zhang et al. 2006).

Differential methylation and its silencing effects on nearby genes have been widely reported in rice in relation to drought, salinity, cold, and oxidative stresses. Methylation signatures appear to be genotype-specific with significant changes between stress and recovery in either transgenerational or non-transgenerational manner (Garg et al. 2015; Xia et al. 2016; Zheng et al. 2013). While stress-induced differential DNA methylation in response to stress has been observed in rice, direct links to their causal siRNAs have not been fully investigated. Experiments from *OsDCL3* mutants confirmed that most of candidate siRNAs are biological 24 nt siRNAs derived from Miniature Inverted-repeat Transposable Elements (*MITEs*) (Sunkar and Zhu 2004; Wei et al. 2014).

MITEs are well distributed in euchromatic regions of the rice genome, residing in almost 58% of protein-coding genes, and associated with 23% of sRNA (Jiang et al. 2011; Lu et al. 2012; Naito et al. 2009; Song and Cao 2017). An increasing number of studies support the key roles of *MITEs* in siRNA generation. For instance, the cold-induced *siR441* and *siR446* in rice originate from *MITE* loci and involved in modulating ABA signaling by targeting the F-box domain protein *OsMAIF1* (Yan et al. 2011). *MITEs* are also involved in RdDM, and their insertion within the promoter of *RAV6* has been shown to provide methylation sites that affect components of brassinosteroid signaling (Song and Cao 2017; Zhang et al. 2015). Given the dominant presence of *MITEs* in the rice genome, there is a growing appreciation of their potential contributions to cold and water-deficit response mechanisms by virtue of their involvement in epigenetic regulation of the transcriptome through siRNAs and RdDM or through rapid sequence evolution. Our survey of a small subset of *MITE*-affected loci in the rice genome revealed two interesting hypotheses (Fig. 17.6). For instance, a subset of genes relevant to the mechanisms of cold stress (Table 17.1) is likely to be affected by *MITE*-induced RdDM, as suggested by potential *MITE* footprints across their coding and upstream regions, consistent with their downregulation by cold. Conversely, a subset of cold-upregulated genes appeared to have acquired cold stress-related *cis*-elements possibly through *MITEs* (Naito et al. 2009).

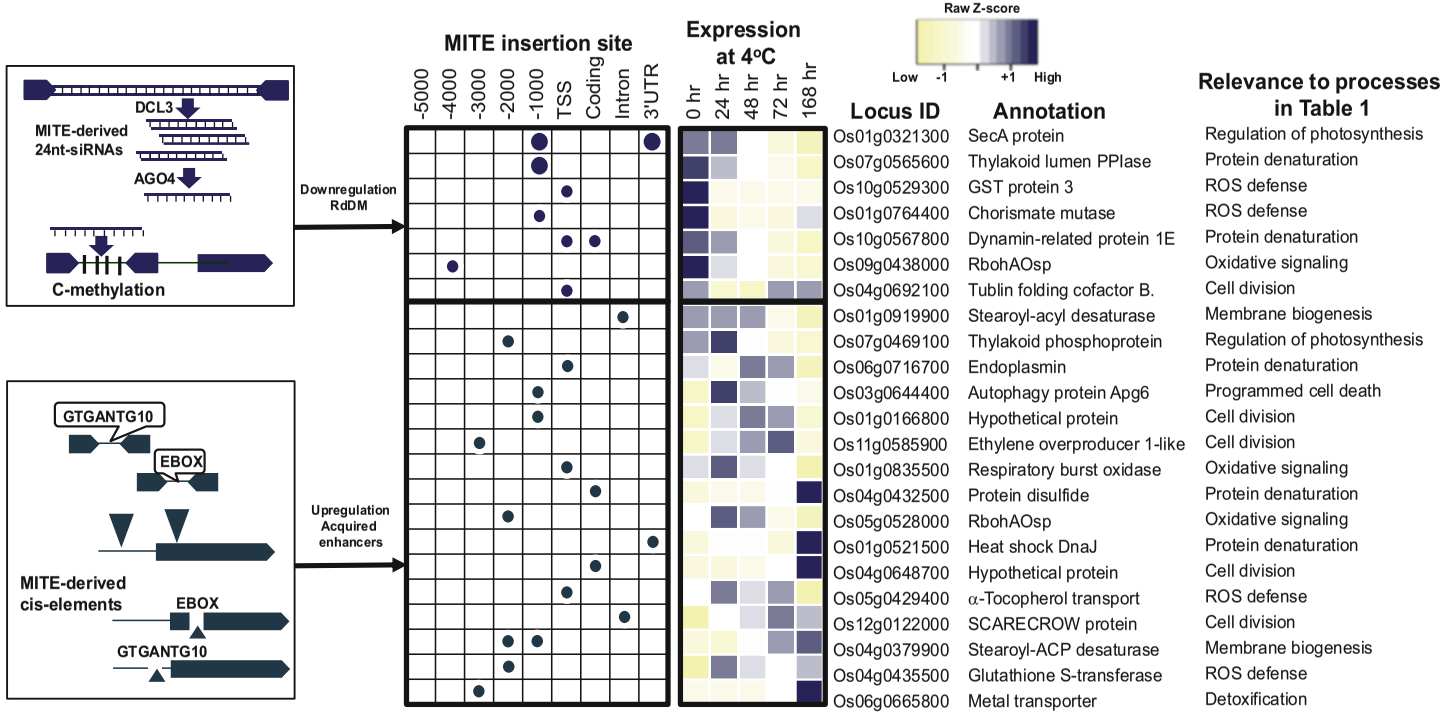

Fig. 17.6 Conceptual model supporting the hypothesis on the possible roles of the Miniature Inverted-repeat Transposable Elements (*MITEs*) in reconfiguring the transcriptome of rice under cold stress. The siRNA loci with *MITE*-derived sequences could be the sites of siRNA generation and target sites for RdDM, causing TGS across the locus. Our analysis of several genomic loci with possible *MITE* insertions within the coding or upstream regions in the Nipponbare

Fig. 17.6 (continued) reference genome revealed that the loss of cold-inducible expression of some cold-regulated genes is possibly due to changes in DNA methylation (*unpublished data by these authors*). Many genes affected by this process are associated with various types of injury and defense mechanisms such as ROS defenses, regulation of photosynthesis, oxidative signaling, and cell growth (Table 17.1). Activation of *MITEs* by stress may also lead to their insertion within promoter regions, leading to the acquisition of sequences including new cis-elements. The presumed *MITE* insertion within upstream regions of certain rice genes may have led to gains of stress-associated cis-elements such as GTGANTG10 and EBOX. Our analysis of these *MITE*-inserted genes showed that many of them are upregulated by cold (*unpublished data by these authors*)

17.5 Quantitative Trait Loci for Cold and Dehydration Tolerance

17.5.1 Cold Tolerance

Several QTL for seedling vigor as a function of shoot, root, coleoptile, and mesocotyl growth at 18 °C were the first to be identified in rice (Mackill and Lei 1997; Redona and MacKill 1996). With the rapid advances in genotyping technology, the list of QTL associated with cold tolerance continues to grow (Table 17.2). However, a major limitation of the current approaches to QTL mapping for cold tolerance is that most if not all of the QTL were identified through the segregation of nonphysiological or non-biochemical traits such as germination efficiency and seedling growth for seedling-stage cold tolerance and spikelet, pollen, and seed fertility for reproductive-stage cold tolerance (Andaya and Mackill 2003a, b; Andaya and Tai 2007; Fujino et al. 2008; Saito et al. 2010). More recent efforts revealed a total of 36 regions across 11 chromosomes with significant QTL effects on a number of nonphysiological traits at the flowering stage, including days to heading, panicle length, panicle exertion, panicle number, spikelet fertility, and grain weight (Jiang et al. 2003; Shinada et al. 2013). Furthermore, based on a study of a diversity panel of 295 rice cultivars from diverse ecological origins, it is estimated that as many as 70 QTL determine seedling cold tolerance potential in rice (Wang et al. 2016). No extensive studies on pyramiding cold tolerance QTL have so far been conducted to assess their cumulative effects.

17.5.2 Dehydration Tolerance and Yield Stability Under Drought

The precise nature of drought tolerance phenotype in rice had long been debated, but earlier efforts on QTL discovery focused on the contributions of root growth and morphology to water use efficiency (Lou et al. 2015; Uga et al. 2013). QTL for root length and angle and soil surface rooting have large effects on plant survival under water deficit (Table 17.2). In the past, grain yield was not considered appropriate for drought tolerance selection due to its low heritability. However, selection for root traits alone did not lead to as much yield advantage than what was initially expected. Positive correlations between yield potential and yield stability under drought were subsequently established (Kumar et al. 2009, 2014; Vikram et al. 2011). Thus, identification of large-effect QTL has recently focused on grain yield penalty as negative effects to photosynthesis, metabolism, and source-sink potential (Courtois et al. 2003; Dixit et al. 2012; Gu et al. 2012; Vikram et al. 2011). The general trend that emerged from physiological studies was that the long-term components of drought tolerance represent the capacity to minimize penalty to grain yield, while the short-term components represent the

Table 17.2 Partial list of cold and dehydration tolerance QTL identified in rice during the last 20 years

Relevant phenotypic trait	Chromosome (*QTL*)	Effect on stress tolerance
Early seedling growth	**2** (*qCTS-2*)	Reduced seedling cold injury
	3 (*qSV-3-1*, *qSV-3-2*)	Reduced seedling cold injury
	4 (*qCTS4*, qCTS4a, qCTS4b)	Reduced seedling cold injury
	5 (*qSV-5*)	Reduced seedling cold injury
	8 (*qSV-8-1*, *qSV-8-2*)	Reduced seedling cold injury
	11 (*qCtss11*)	Reduced seedling cold injury
	12 (*qCTS12*, *qCTS12a*)	Reduced seedling cold injury
Seed of germination	**2** (*qLVG2*)	Good seedling vigor under cold
	3 (*qLTG3-1*)	Good seedling vigor under cold
	5 (*qCTB-5-1*, *qCTB-5-2*)	Cold tolerance at bud burst stage
	7 (*qLVG7-2*, *qCIVG7-2*, *qCTB-7*)	Cold tolerance at bud burst stage
	11 (*qCTP11*)	Cold tolerance at plumule stage
	12 (*qCTP12*)	Cold tolerance at plumule stage
Reproductive growth	**3** (*qPSST-3*)	Pollen fertility and yield as affected by cold
	7 (*qPSST-7*)	Pollen fertility and yield as affected by cold
	9 (*qPSST-9*)	Pollen fertility and yield as affected by cold
Spikelet fertility	**1** (*qCTB-1-1*)	Cold tolerance at booting stage
	4 (*Ctb1*, *qCTB-4-1*, *qCTB-4-2*)	Cold tolerance at booting stage
	5 (*qCTB-5-1*, *qCTB-5-2*)	Cold tolerance at booting stage
	10 (*qCTB-10-1*, *qCTB-10-2*)	Cold tolerance at booting stage
	11 (*qCTB-11-1*)	Cold tolerance at booting stage
Seed fertility	**3** (*qLTB3*)	Yield stability as affected by cold stress
Root growth angle, soil surface rooting	**7** (*DRO3*, *qSOR1*)	Drought avoidance by effective root growth
Root growth angle	**9** (*DRO1*)	Drought avoidance by effective root growth

List includes only the *named* QTL affecting cold tolerance at seedling and flowering stages and those affecting drought tolerance as a function of root growth. Other chromosomal regions where QTL effects have been detected but QTL have not been named are not included in this list

capacity for vegetative injury prevention. Germplasm screening identified a core set of donors for large-effect QTL for yield stability (*qDTY*). These include a number of traditional cultivars used in IRRI's breeding program (Table 17.3) (Ghimire et al. 2012; Kumar et al. 2014; Mishra et al. 2013; Sandhu et al. 2014; Vikram et al. 2015). Multilocation and multiyear phenotyping facilitated the fine-mapping of several large-effect *qDTYs*. However, a caveat on the use of

Table 17.3 List of drought tolerance-associated QTL identified at IRRI during the last decade

QTL	Original source/donor	Recipient cultivar	Optimal ecosystem
$qDTY_{1.1}$	N22, Dhagaddeshi, Apo, CT9993-10-1 -M, Kali Aus, Basmati 334	Swarna, IR64, MTU1010	Lowland, upland
$qDTY_{2.1}$	Apo, Aus276	Swarna, MTU1010	Lowland
$qDTY_{2.2}$	Aday Sel, Kali Aus	MTU1010, IR64, Samba Mahsuri	Lowland, upland
$qDTY_{2.3}$	Vandana, N22, IR74371-46-1-1, Kali Aus	IR64, Sabitri, Way Rarem	Lowland, upland
$qDTY_{3.1}$	Apo, IR55419-04	Swarna, TDK1	Lowland
$qDTY_{3.2}$	Vandana, N22, IR77298-5-6-18, IR74371-46-1-1, Moroberekan	Way Rarem, Swarna, Sabitri	Lowland, upland
$qDTY_{4.1}$	Aday Sel	IR64, Samba Mahsuri	Lowland
$qDTY_{6.1}$	Apo, Vandana, IR55419-04	Swarna, IR72, TDK1	Upland, lowland
$qDTY_{6.2}$	IR55419-04	TDK1	Lowland
$qDTY_{9.1}$	Aday Sel	IR64	Lowland
$qDTY_{10.1}$	N22, Aday Sel, Basmati 334	IR64, MTU1010, Swarna	Lowland
$qDTY_{12.1}$	Way Rarem, IR74371-46-1-1	Vandana, Sabitri	Upland, lowland

QTL have established large effects on grain yield stability as a function of tolerance to episodic drought during the critical stages of reproductive growth. First digit of the QTL subscript represents chromosomal location

these donors in cultivar enhancement is that many of them contribute to phenotypic drags because of undesirable traits such as photoperiod sensitivity.

17.5.3 Drought Tolerance QTL Pyramiding, Linkage Drag, and Gene Interaction

Tight association of QTL markers with yield stability streamlines the selection process through high-resolution profiling of foreground and background genomes across combinations of donor and recipient parents. At IRRI, marker-assisted selection generated a number of elite varieties, which exhibit many of the desired background traits such as early maturity, ideal root length, density and plasticity, ideal shoot growth, and water-nutrient-use efficiency in addition to a yield advantage of 0.8–1.0 tons per hectare (Vikram et al. 2015). However, given the genetic backgrounds of the QTL donors, negative effects of linkage drag, pleiotropic functions, and/or negative epistatic interactions are major challenges affecting the rate of successful QTL deployment (Li et al. 2003). Fine-mapping of $qDTY_{1.1}$ resolved the linkage drag between the beneficial alleles contained within the QTL

and the proximal recessive allele for the *sd1* gene for tall stature. Likewise, $qDTY_{2.3}$, $qDTY_{3.1}$, $qDTY_{3.2}$, and $qDTY_{12.1}$ are also proximally located to other loci with undesirable alleles for late flowering, tall stature, or poor yield (Vikram et al. 2011, 2015, 2016).

The QTL pyramiding strategy promises to optimize the most desirable combination(s) of traits across genetic backgrounds for maximum possible effects. At IRRI, the major emphasis is the deployment of $qDTY_{1.1}$, $qDTY_{2.2}$, $qDTY_{3.1}$, $qDTY_{3.2}$, $qDTY_{6.1}$, and $qDTY_{12.1}$ (Kumar et al. 2014). Since these QTL exhibited stable phenotypic effects, it was hypothesized that incremental yield advantage under drought could be achieved with QTL pyramids, commensurate with the predicted cumulative effects of each QTL. Contrary to expectations, pyramided lines did not always show the projected positive effects. Rather, differential reaction of individual QTL or combinations of QTL across genetic backgrounds was a common observation. Interaction ranged from reduced effect of individual QTL in certain genetic backgrounds to enhanced effects of the same QTL in other genetic backgrounds. Similar trends were observed for various combinations of two or more QTL across genetic backgrounds (Henry et al. 2015; Sandhu et al. 2014; Shamsudin et al. 2016). The negative QTL × QTL or QTL × genetic background interactions that led to phenotypic drags have been addressed by trial and error to reveal the most ideally complementing combinations. For example, combinations of $qDTY_{2.3}$ and $qDTY_{3.2}$ with $qDTY_{12.1}$ had greater propensity for positive effects across genetic backgrounds compared to other combinations (Dixit et al. 2012; Shamsudin et al. 2016). Negative complementation, disrupted synergy, or antagonistic interactions among QTL and between QTL and other genes/alleles in the genetic background have been hypothesized as possible causes (Courtois et al. 2003; Ghimire et al. 2012).

17.5.4 Linking QTL to Networks of Cellular Processes

Maximizing the full potential effects of QTL is all about optimal interactions that can be understood by direct links between QTL and genetic networks. The major problem is that there are no concerted efforts for systematic linking of the outputs of QTL mapping and functional genomics in rice. This major gap is illustrated by the fact that none of the traits used for mapping cold and drought tolerance QTL were physiological or biochemical in nature relevant to the known mechanisms of defenses that were uncovered by functional genomics. Without such links, it becomes difficult to evaluate which among the various types of stress injuries and their associated defense or avoidance mechanisms have either essential or negligible contributions to maximum stress tolerance potentials, especially in context of yield penalty (Table 17.1; Figs. 17.3, 17.4, and 17.5). While there are growing efforts to translate QTL data into biochemical mechanisms with candidate gene analysis, the real disconnect is that much of those efforts tend to oversimplify the functional significance of QTL to just a single biochemical or regulatory gene within a QTL (Dixit et al. 2015; Mohanty et al. 2016). This becomes a real major

issue in elucidating genetic mechanisms given that the biochemistry and physiology of stress tolerance, especially in context of penalty to yield, cannot be explained simply by just one protein or by a single biochemical process. Analysis and interpretation of the mechanistic aspects of QTL effects should be based on networks of biochemical and regulatory pathways that could involve QTL × QTL or QTL × genetic background complementation (see also Chaps. 10, 11, 12, 13, 14, 15, and 16).

To maximize the effects of QTL pyramids, it is very critical to understand the genic and/or allelic content across the genomic background. Even large-effect QTL explain only a portion of the total phenotypic variation attributed to genetic effects, indicating that lots of other entities are essential for synergistic or additive effects. Despite these observations, a reductionist view still exists that high-resolution QTL maps provide a means to zoom into relatively small genomic region(s) and that validation of one or few candidate genes by functional genomics could directly link the QTL effects to the critical cellular process. This assumption contradicts the likelihood that a network of intricately interacting cellular processes are involved. One recent example is the partitioning of $qDTY_{12.1}$ into sub-QTL, which singled out the $OsNAM_{12.1}$ transcription factor as a master switch for the biochemical genes within the QTL boundary, thereby explaining the effect on yield stability as a function of root traits (Dixit et al. 2015). While these findings represent a significant advance in understanding the likely functions of large-effect QTL, the vision of such a paradigm is very limited because it did not address how the QTL function in terms of their interactions with other minor components in the genetic background.

The $qDTY_{12.1}$ is known to exhibit negative interactions with other QTL and genetic background. This should prompt a more critical reevaluation of the current logic behind the reductionist view of the regulatory, biochemical, and physiological aspects of QTL functions as one master switch. Similar studies correlating QTL for natural variation for cold tolerance in *Arabidopsis* have shown that the master switch CBF/DREB factors did not explain the large-effect QTL. Conversely, known QTL for cold and dehydration tolerance did not fully explain the positive effects of CBF/DREBs as demonstrated by functional genomics (Alonso-Blanco et al. 2005). Therefore, genes or alleles contained within a QTL should be viewed as important components of a network that configures the full phenotypic potential, by virtue of their interactions among themselves and with other genes or alleles in the genetic background.

17.6 Conclusions: Cold and Drought Tolerance Are Multidimensional and Defined by Intricate Interactions and Optimal Regulatory Networks

Independent efforts by agronomists, physiologists, cell biologists, and biochemists revealed many critical biochemical and physiological processes involved in the multidimensional complexity of stress injury and defenses at the cell, tissue/organ,

and whole-plant levels (Fig. 17.1). Modern paradigms of functional genomics have placed regulatory transcription factors, signaling and transport proteins, and even noncoding RNAs in context of the regulation of cellular stress physiology (Figs. 17.2, 17.3, 17.4, 17.5, and 17.6; Table 17.1). Independent efforts by rice breeders using genomics-assisted classical genetic studies identified several large-effect QTL for optimal vegetative growth and grain yield under stress environments (Tables 17.2 and 17.3).

Despite these important milestones, rice breeders are still facing major bottlenecks due to the limitations of one-dimensional approach to the complex multidimensional nature of stress tolerance potential. The clear consensus from functional genomics is that transgenic manipulation of one or few regulatory and/or metabolic genes could lead to enhanced stress tolerance in terms of reduced injury and better plant survival but limited impact to minimizing penalties to net growth and yield. Incremental gains in cell, tissue/organ, or whole plant-level stress tolerance that resulted from single-gene manipulation are often undermined by negative pleiotropic effects. There is no clear consensus on how the regulatory and metabolic genes identified by functional genomics correlate with the large-effect QTL. Therefore, the message from functional genomics is that stress tolerance potential requires the optimization of regulatory network configurations for balancing vegetative survival and growth with efficient allocation of resources to support positive net gains in yield.

The QTL strategy has identified large-effect genomic components that translate to incremental yield advantage under stress conditions, especially for drought tolerance. The current biggest challenge for the utilization of these QTL for pyramiding is the fact that very little is known on how they function in the context of genetic networks. A given QTL does not act alone to confer the full phenotype, and because the nature of interactions are not known, it is difficult to predict optimal effects across combinations. Nevertheless, current data supports the hypothesis that optimal genetic interactions could be achieved by intra-QTL or inter-QTL complementation, as well as through ideal interactions between QTL genes and other essential alleles in the genetic background. These interactions presumably determine whether the regulatory network is optimal or not.

The conceptual models depicted in Fig. 17.7 illustrate an integrated view of how the outcomes of functional genomics and QTL mapping could be integrated toward understanding the nature of genetic interactions that lead to maximum stress tolerance potential. Maximum stress tolerance potential comes from the integration of different dimensions of cellular and whole-plant level processes and adjustments (D_1, D_2, D_3), which address the different layers of perturbation in a *domino effect* fashion (Fig. 17.7a). Each of these dimensions may be comprised of many sub-networks ($D_{1.1}$, $D_{1.2}$, $D_{1.n}$), representing different processes or gene regulons, defined by the regulatory or biochemical genes that control them. Therefore, stress tolerance potential represents the cumulative effects of the three dimensions and ideal combinations of genes or alleles that control them.

Most rice genotypes are expected to exhibit the D_1 mechanisms as general or first line of defense. However, only certain rice genotypes have the capacity to

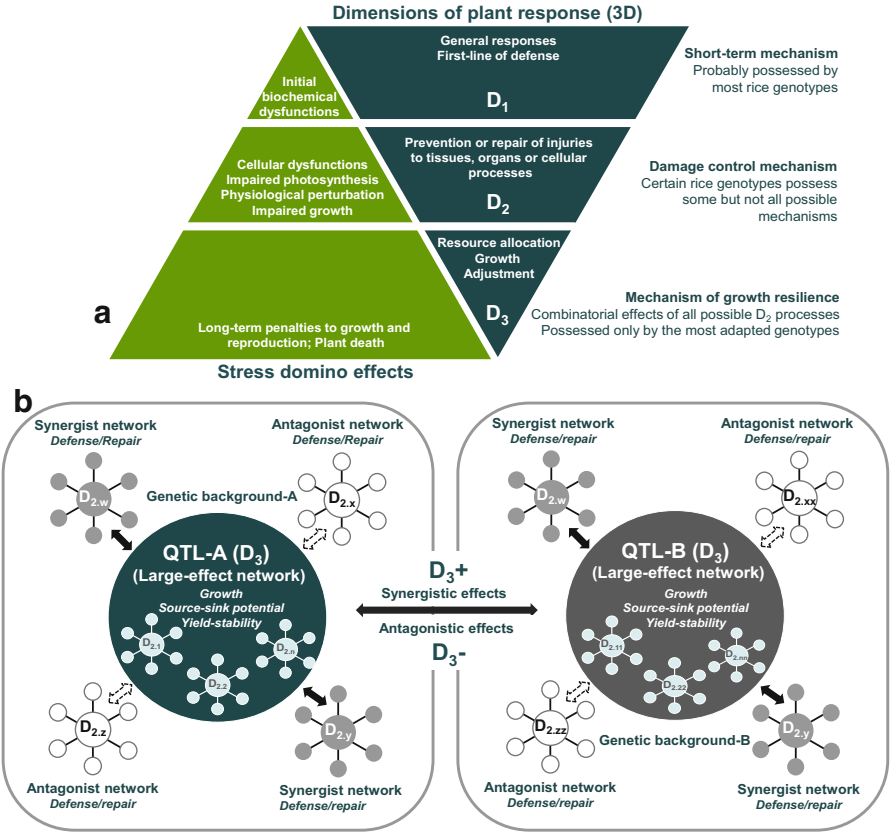

Fig. 17.7 Conceptual models illustrating the integration of different dimensions of cellular and whole-plant level responses to physiological perturbations during cold and water deficit. (**a**) Inverse pyramid connecting the three dimensions of plant responses to cold and water deficit with different levels of perturbation during the progression of stress *domino effects*. Reciprocal triangle on the right represents the nature of physiological and genetic mechanisms (D_1, D_2, D_3) corresponding to each level of perturbation. Maximum stress tolerance potential depends on the cumulative effects of the three dimensions. (**b**) Model translating the inverse pyramid concept in context of genes, quantitative trait loci, genetic backgrounds, and their interactions. Different types of biochemical/physiological response mechanisms or gene regulons under the D_2 dimension may be encoded as major interacting subnetworks within a QTL, hence large-effects. They may also be encoded by other networks of defense/repair-related genes in the genetic background. Maximum stress potential depends on the nature of synergy or antagonism among QTL or between QTL and genetic background

proceed one step further to execute the next level of specialized mechanisms (D_2) that could address some but not all types of injuries, hence limited protection from yield penalty. D_2 processes are configured likely at the expense of diverting cellular resources from growth-related responses. D_3 processes are consequences of ideal integration of several combinations of D_2 processes with other aspects of metabolic adjustment that positively affect nutrient assimilation, transport, partitioning, and

most importantly photosynthesis. These mechanisms are important for maximizing net gains despite the large diversion of metabolic intermediates for defense. The predicted cumulative outcome of the synergies between D_2 and D_3 mechanisms is less yield penalty from stress. Since most D_2 mechanisms do not seem to co-localize with large-effect QTL, they could either act as *synergist* by positive complementation/epistasis or as *antagonist* by virtue of their physiological drags to the effects of D_3 (Fig. 17.7b). It is imaginable that coupling or uncoupling of compatible and incompatible genes/alleles or mechanisms as a consequence of combining different types of D_3 and/or combining D_3 with D_2 could determine the potential for optimal or significantly reduced functionality of genetic networks. Thus, the mechanisms configured by one QTL or by the genetic background could either complement, enhance, fine-tune, downplay, or antagonize the mechanisms configured by other QTL or other genes in the genetic background.

Functional and/or regulatory compatibilities among QTL across genetic backgrounds appear to be critical in defining the potential for optimal networks. The culprit could be changes in gene expression as a consequence of either optimal or suboptimal interactions. With the available data from QTL mapping, functional genomics, physiology, and biochemistry, it may be an opportune time to consider network engineering as a guiding principle for enhancing yield stability under cold and water deficit in rice. A series of large-effect QTL combinations across a spectrum of genetic backgrounds with different defense mechanisms or regulons could be created and interrogated with the following questions: *Does a given QTL affect the expression of other genes in certain genetic backgrounds or* vice versa*? What are the physiological implications of such interactions, and do they reveal the nature of the networks that lead to synergistic or antagonistic effects? What yield-associated QTL tend to be enhanced or negated by its co-occurrence with what type of defense mechanisms known from functional genomics? Could we use such correlation to model the network configurations for maximum positive complementation with less trade-offs?*

With these questions, it is possible to unravel the nature of regulatory and biochemical networks and identify the pathways that could be engineered together in the same genetic background to minimize trade-offs. The power of comparative genome resequencing, transcriptome profiling at both mRNA and ncRNA levels, methylome sequencing, metabolome profiling, and molecular interactome data must be exploited across a genetic series representing combinations of QTL with other network hubs and across a spectrum of genetic backgrounds. Advanced technology for integrating multiple dimensions of investigation through sophisticated biocomputing and novel algorithms will be very critical in revealing biologically meaningful patterns. Identification of multiple donors of compatible network interactions will also be critical.

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Chapter 18
Pathogen Recognition and Immune Signaling

Tsutomu Kawasaki

Abstract Rice blast and bacterial blight, two important rice diseases, are caused by infection with a fungal pathogen *Magnaporthe oryzae* and a bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), respectively. Recent studies on the interaction between rice and these pathogens provided important knowledges of the molecular mechanisms of rice immune responses such as receptor-mediated pathogen recognition, host immune signaling, and pathogen effector-mediated susceptibility. So far, many disease resistance (*R*) genes have been genetically identified based upon disease resistance traits against *M. oryzae* and *Xoo*. Most rice blast *R* genes isolated to date encode pathogen recognition receptors. In contrast, the majority of bacterial blight *R* genes are involved in transcriptional regulation of host resistance or susceptibility factors. Genetic and biochemical studies of rice immune signaling have identified important immune factors including OsRac1, OsRLCK185, and WRKY45. Identification of rice factors that interact with OsRac1, OsRLCK185, and WRKY45 revealed the molecular mechanisms of a variety of immune responses, including the expression of defense-related genes, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK), and lignification.

Keywords Disease resistance gene · Pattern recognition receptor · NB-LRR receptor · Pathogen effector · GTPase · RLCK · MAPK · Reactive oxygen species · Lignification · WRKY

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18.1 Introduction

Plants have evolved the ability to recognize and defend against infection of pathogens. Recognition of pathogens activates intracellular immune signaling mediated by protein kinases, G proteins, and transcription factors, which control a series of immune responses including the synthesis of antimicrobial compounds, cell wall reinforcement, and production of reactive oxygen species (ROS). To overcome host defenses, pathogens deliver a variety of effectors into host cells. The effectors target host factors to inhibit the induction of host immune responses or alter nutrition distribution suitable for pathogen proliferation. To counteract these effectors, plants have developed receptors to recognize the effectors. Plants also possess an immune system against secondary infection of pathogens, referred to as systemic acquired resistance (SAR), which confers long-lasting protection against a broad spectrum of pathogens. SAR is regulated by the salicylic acid (SA)-mediated pathway. In this chapter, recent progress on the topic of pathogen recognition and immune signaling in rice is reviewed.

18.2 Plant Immune Receptors

Plants have developed a sophisticated two-tier immune system to defend against infection and the growth of pathogens (Fig. 18.1). The first layer of plant immunity is initiated by the perception of pathogen-associated molecular patterns (PAMPs) including fungal chitin and bacterial flagellin and peptidoglycan, by plasma membrane (PM)-localized pattern recognition receptors (PRRs). PRRs are receptor-like kinases (RLKs) with an intracellular kinase domain or receptor-like proteins (RLPs) (Dangl et al. 2013). Both RLKs and RLPs possess a ligand-binding ectodomain and transmembrane domain. The PAMP-induced immunity, referred to as pattern-triggered immunity (PTI), involves transcriptional activation of large numbers of immune-related genes through the rapid activation of MAPKs and the generation of ROS (Dangl et al. 2013), which limits microbial colonization. To overcome host PTI, pathogens deliver effectors into plant cells. The second layer of immunity is induced by direct or indirect recognition of pathogen effectors by host intracellular immune receptors of the nucleotide-binding leucine-rich repeat (NB-LRR) protein family, termed NB-LRR receptors (NLRs). This immunity is termed effector-triggered immunity (ETI). ETI displays a remarkable disease resistance, often accompanied by hypersensitive cell death. Some NLR proteins have been identified as disease resistance (R) proteins encoded by *R* loci, which were defined genetically based on disease resistance traits (Dangl et al. 2013).

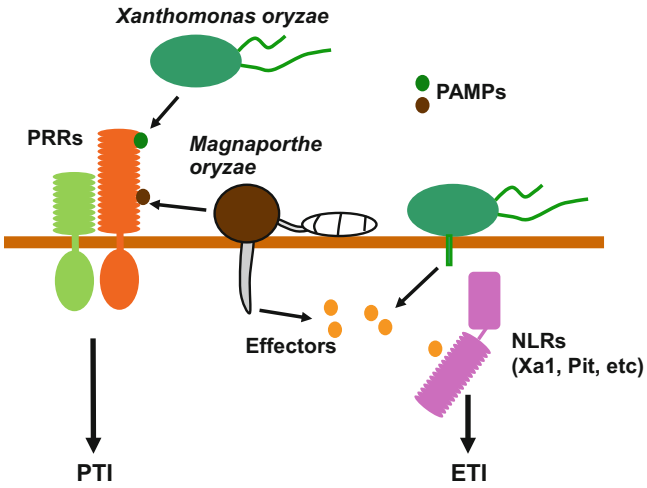

Fig. 18.1 PRR- and NLR-mediated pathogen recognition. PRRs recognize pathogen-associated molecular patterns including fungal chitin and bacterial flagellin. Pathogens deliver effectors into plant cells, which are perceived by NLRs

18.2.1 Rice R *Genes*

Rice blast and bacterial blight diseases are most serious diseases for rice production. So far, 25 *R* genes against rice blast have been identified (Liu et al. 2014). Among these, 24 of these rice blast *R* genes encode NLRs, which recognize corresponding pathogen effectors inside plant cells.

Based on genetic analyses of bacterial blight resistance, 41 *R* genes have been registered in the Oryzabase database (http://shigen.nig.ac.jp/rice/oryzabase/gene/list). Twenty-nine *R* genes are dominant and 12 are recessive. Among these, ten genes (*Xa1*, *xa5*, *Xa10*, *xa13*, *Xa21*, *Xa23*, *xa25*, *Xa26/Xa3*, *Xa27*, and *xa41*) have been isolated. *Xa1* encodes an NLR (Yoshimura et al. 1998). Xa21 and Xa26/Xa3 are LRR-RLKs (Zhang and Wang 2013). *xa13*, *xa25*, and *xa41* are caused by loss-of-function mutations of the *SWEET* genes, which belong to a newly identified family of sugar transporters (Zhang and Wang 2013). Expression of their corresponding functional genes *Xa13*, *Xa25*, and *Xa41* was enhanced by transcription activator-like (TAL) effectors of *Xoo* (Fig. 18.2; see Chap 19 for details), which may lead to supply sugars to the pathogens. A limited transportation of sugar, which was caused by the *xa13*, *xa25,* and/or *xa41* mutations, resulted in enhanced resistance to bacterial blight disease.

Xa10, Xa23, and Xa27 are novel proteins with unknown functions (Zhang et al. 2015). Xa10 shares approximately 50% amino acid sequence identity with Xa23. Expression of these three genes was also induced by TAL effectors. However, in contrast to the *SWEET* genes, expression of *Xa10*, *Xa23*, and *Xa27* induced resistance to *Xoo* (Fig. 18.2). Therefore, these genes have been referred to as

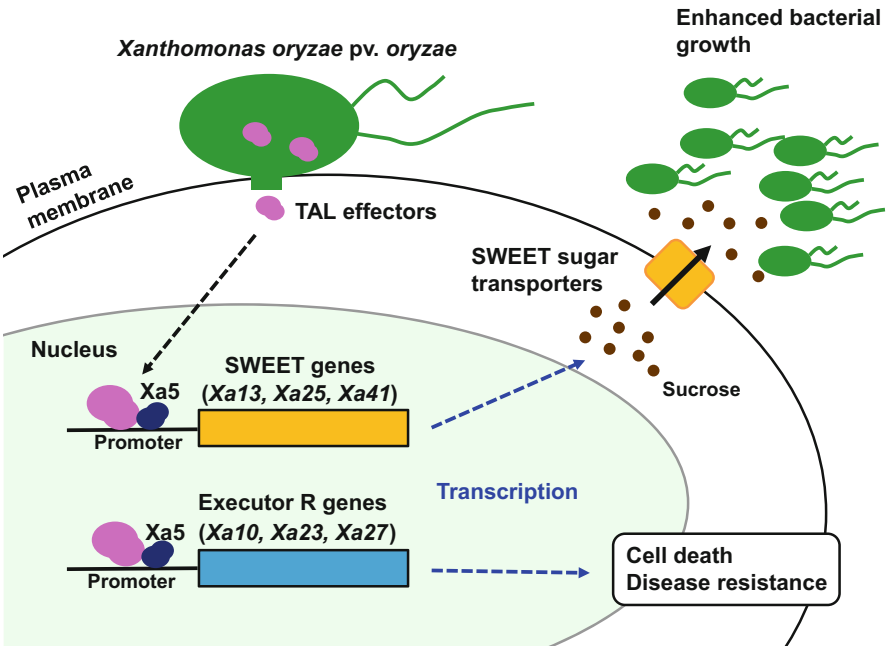

Fig. 18.2 *Xoo* TAL effector-mediated expression of *SWEET* and executor *R* genes in rice. TAL effectors are secreted into rice cells and move to nucleus using their nuclear localization signals. TAL effectors bind to cis-elements in the promoters of *SWEET* and executor R genes and activate their transcription. TAL effector-mediated transcription requires Xa5. Expression of *SWEETs* encoding sugar transporters facilitates diffusion of sucrose across plasma membranes, which results in increased sugar availability of *Xoo*. In contrast, expression of executor *R* genes such as *Xa10*, *Xa23*, and *Xa27* induces cell death and resistance to *Xoo*

"executor *R* genes." The *Xa5* dominant allele encodes the transcription factor IIA gamma subunit 5 (TFIIAγ5). A direct interaction between Xa5 and TAL effectors is required for the expression of *Xa23* and susceptibility genes including the *SWEET* genes (Yuan et al. 2016). Because the *xa5* mutant protein loses the ability to interact with TAL effectors, the TAL effectors are not able to induce the expression of susceptibility genes in *xa5* mutant plants.

18.2.2 Rice PRRs

Rice contains more than 1100 RLKs/RLPs (Shiu et al. 2004), which are predicted to be involved in immunity and development. Xa21, an RLK with an extracellular LRR, was initially identified as an R-protein for bacterial blight resistance (Song et al. 1995), because it induces robust defense responses including HR-like cell death. However, the ligand for Xa21 was recently reported to be a sulfated *Xoo*

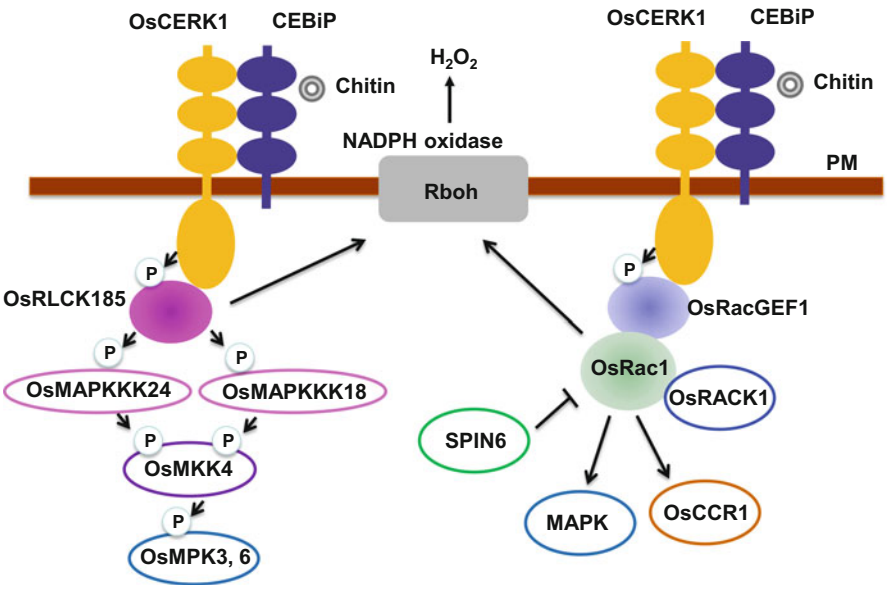

Fig. 18.3 Chitin-activated immune signaling in rice. Upon perception of chitin, OsCERK1 phosphorylates OsRLCK185 and OsRacGEF1, which trigger activation of the MAPK cascade and OsRac1. OsRac1 directly regulates the activities of OsCCR1 and ROS-generating NADPH oxidase (Rboh)

protein RaxX, which is conserved in many pathogenic *Xanthomonas* species (Pruitt et al. 2015), suggesting that Xa21 is a PRR. Xa21 requires co-receptor OsBAK1 for recognition of the ligand (Chen et al. 2014), which is consistent with the fact that *Arabidopsis* PRRs, FLS2, and EFR for bacterial flagellin and elongation factor-Tu, respectively, associate with BAK1 in a ligand-dependent manner (Liebrand et al. 2014). In addition, the ubiquitin E3 ligase XB3, the protein phosphatase XB15, and ATPase XB24 interact with Xa21 (Chen et al. 2010c), which are required for proper control of Xa21-mediated immune signaling.

Among PRR-mediated immune responses in rice, fungal chitin-induced immune signaling has been well investigated as a model system for rice PTI (Fig. 18.3). Rice chitin receptor OsCEBiP is a PM-localized RLP with an extracellular lysin motif (LysM) domain (Kaku et al. 2006). The LysM domain directly binds to chitin and subsequently interacts with a LysM-RLK-type co-receptor OsCERK1 (Shimizu et al. 2010). OsCERK1 phosphorylates the receptor-like cytoplasmic kinase (RLCK) family protein OsRLCK185 and the guanine-nucleotide exchange factor OsRacGEF1 (Fig. 18.3) (Yamaguchi et al. 2013; Akamatsu et al. 2013), which activates intracellular immune signaling. A U-box ubiquitin E3 ligase OsPUB44 was also reported to play an important role in chitin signaling (Ishikawa et al. 2014). In addition to chitin recognition, OsCERK1 also participates in the perception of bacterial peptidoglycan (PGN) by interaction with two LysM-RLP-type PGN

receptors LYP4 and LYP6 (Kouzai et al. 2014). In fact, OsCERK1 regulates defense responses against both fungal pathogen *M. oryzae* and bacterial pathogen *Xoo* (Kouzai et al. 2014).

18.2.3 Rice NLRs

Most rice blast R-proteins are intracellular NLRs. Rice blast resistance is often mediated by paired NLRs that are tandemly clustered in the genome. For example, the NLR pair *RGA4* and *RGA5* are encoded by the *Pi-CO39*/*Pia R* locus (Cesari et al. 2014). In the pair, RGA4 functions as the inducer for disease resistance and cell death, which is suppressed by RGA5 in the absence of the pathogen. RGA5 recognizes the *M. oryzae* effectors AVR-CO39 and AVR-Pia through direct binding of the effectors to the RATX1 (Related to ATX1) domain of RGA5. The recognition of the effectors by RGA5 leads to derepression of RGA4, which results in the activation of immune responses. The RATX1/HMA domain was also found in rice blast R-protein Pi-k, and it interacted directly with the *M. oryzae* effector AvrPi-k (Kanzaki et al. 2012). Of note, Pi21 containing the RATX1/HMA domain functions as a susceptibility factor for rice blast. In fact, a loss-of-function mutation of *Pi21* reduces disease symptoms of the fungus (Fukuoka et al. 2009). These results suggested that rice blast fungus somehow uses host RATX1/HMA proteins for its virulence, and plants integrate the RATX1/HMA domains into NLR proteins as decoy domains to detect blast infection.

As mentioned above, *Xa1* encodes the only NLR among *R* genes against bacterial blight. Recently, Xa1 was reported to recognize *Xoo* TAL effectors (Ji et al. 2016), which induce ETI. However, the molecular mechanism of how Xa1 recognizes the corresponding TAL effectors remains to be analyzed.

18.2.4 RLCK-Mediated Immune Signaling

Rice contains 379 receptor-like cytoplasmic kinase (RLCK) proteins (Vij et al. 2008), which are classified into 13 subfamilies (RLCK I–XIII). Recent reports indicated that RLCK VII family proteins interact with the intracellular kinase domains of RLKs and are involved in ligand-dependent intracellular signaling (Couto and Zipfel 2016). OsRLCK185 was identified as a target of the *Xoo* XopY (Xoo1488) effector, which suppresses rice immunity (Yamaguchi et al. 2013). OsRLCK185 is phosphorylated by OsCERK1 at the PM in a ligand-dependent manner, which was inhibited by XopY. OsRLCK185 regulates early signaling immune responses such as MAPK activation and ROS production. In addition, OsRLCK57, OsRLCK107, OsRLCK118, and OsRLCK176 were reported to play roles in chitin-induced immunity (Ao et al. 2014; Li et al. 2017). Recently, we found that OsRLCK185 interacts with and phosphorylates OsMAPKKK18, 1 of

75 rice MAPKKKs, which triggers chitin-induced activation of MAPKs, OsMPK3, and OsMPK6, in rice (Yamada et al. 2017). In rice, chitin-induced activation of these MAPKs is known to be regulated through a MAPKK, OsMKK4 (Kishi-Kaboshi et al. 2010). In fact, OsMAPKKK18 phosphorylates OsMKK4 (Yamada et al. 2017). In addition, OsMAPKKK24 was also reported to be regulated by OsRLCK185 (Wang et al. 2017). Thus, it is likely that OsRLCK185 plays major roles in chitin-induced MAPK activation. Recently, we found that the chitin-induced MAPK signaling pathways are conserved in rice and *Arabidopsis* (Yamada et al. 2016, 2017).

In addition to the activation of MAPK cascades, OsRLCK185 regulates chitin-induced ROS production. In *Arabidopsis*, the FLS2-associated RLCK VII BIK1 activates a PM-localized NADPH oxidase, AtRbohD, by phosphorylation of its N-terminal region, which triggers flagellin-induced ROS production (Kadota et al. 2014). Therefore, it is possible that OsRLCK185 may regulate rice Rboh activity in similar way to BIK1.

BSR1 (OsRLCK278), a member of the RLCK VII family, was identified by the fact that overexpression of BSR1 enhanced disease resistance in *Arabidopsis*. Transgenic rice plants overexpressing BSR1 displayed resistance to four different pathogens, a fungal pathogen *M. oryzae*, two bacterial pathogens *Xoo* and *Burkholderia glumae*, and a rice stripe virus (Maeda et al. 2016). However, the molecular mechanism of how BSR1 regulates rice immunity is unknown. Thus, increasing evidence indicates that the RLCK family proteins are important regulators of PRR-mediated immunity.

18.2.5 Activation of OsRac1 by PRRs and NLRs

Plant Rac/Rop small GTPases are conserved in plants and constitute a unique subfamily of the Rho family of small GTPases. These GTPases are regulated by shuttling between a GDP-bound inactive form and a GTP-bound active form. The shuttling is regulated by two regulatory proteins, GDP/GTP exchange factors (GEFs) and GTPase-activating proteins (GAPs). There are seven Rac/Rop proteins in rice (Chen et al. 2010b). Among them, OsRac1 has been well investigated to function as a key regulator in PRR- and NLR-mediated immunity in rice (Kawasaki et al. 1999; Ono et al. 2001; Akamatsu et al. 2013; Kawano et al. 2010). OsRac1 is activated by at least two types of GEFs, OsRacGEF1 and OsSWAP70 (Akamatsu et al. 2013; Yamaguchi et al. 2012). OsRacGEF1 belongs to a plant-specific ROP nucleotide exchanger (PRONE)-type GEF family, whereas OsSWAP70 is a rice homolog of human SWAP70 containing a disuse B-cell lymphoma (Dbl) homology domain as a GEF-catalytic domain.

OsRacGEF1 forms a complex with OsCERK1 (Akamatsu et al. 2015). In response to chitin perception, OsRacGEF1 is phosphorylated by OsCERK1, which is required for the GEF activity toward OsRac1 (Akamatsu et al. 2013). As mentioned above, because OsCERK1 also phosphorylates OsRLCK185 to activate

immune responses (Yamaguchi et al. 2013), there are at least two pathways downstream of OsCERK1, mediated by OsRacGEF1 or OsRLCK185. Whether these two pathways are cooperatively or independently regulated in chitin signaling remains to be identified.

As mentioned above, most rice blast *R* genes encode NLRs. However, how these NLRs transduce intracellular immune signaling is largely unknown. Pit is a PM-localized NLR that induces hypersensitive cell death by recognition of the *M. oryzae* AvrPit effector. The Pit-mediated immune response requires OsRac1, because the resistance was compromised by silencing of OsRac1 (Kawano et al. 2010). Ectopic expression of Pit induces activation of OsRac1 in cells (Kawano et al. 2010). The PM localization of Pit is regulated by palmitoylation of the N-terminal region of Pit (Kawano et al. 2014), which is required for the interaction with and the activation of OsRac1.

18.2.6 OsRac1- and Gα-Mediated Immune Responses

Extensive research on OsRac1-mediated immunity revealed that OsRac1 interacts with a variety of proteins such as NADPH oxidase OsRbohB, Hsp90, Hsp70, Hop/Sti1, RAR1, the scaffold protein OsRACK1, the lignin biosynthetic enzyme Cinnamoyl-CoA reductase 1 (OsCCR1), and the basic helix-loop-helix transcription factor RAI1 (Wong et al. 2007; Oda et al. 2010; Chen et al. 2010a; Nakashima et al. 2008; Kawasaki et al. 2006; Kim et al. 2012). Hop/Sti1 was originally identified as interacting with OsRac1 (Chen et al. 2010a). Hop/Sti1 forms a cochaperone with Hsp90, which regulates efficient transport of OsCERK1 from the ER to the PM where Hop/Sti1 and Hsp90 form a complex with OsRac1.

ROS induced by pathogen recognition do not only directly kill the pathogens but also function as a second messenger to induce immune responses. OsRac1 directly interacts with the N-terminal region of the PM-localized NADPH oxidase OsRbohB in a GTP-form dependent manner and activates its oxidase (Wong et al. 2007; Oda et al. 2010). In fact, expression of a constitutively active mutant of OsRac1 enhanced ROS production *in planta*. Recently, the interaction between OsRac1 and OsRbohB was reported to occur in PM microdomains that consist mainly sphingolipids and sterols (Nagano et al. 2016). As mentioned above, OsRLCK185 also plays a role in chitin-induced ROS production. Whether OsRac1 cooperates with OsRLCK185 in ROS production remains to be analyzed.

Lignification is important for plant cell wall reinforcement against pathogen attack. Lignin is polymerized through peroxidase activity using H_2O_2 in the cell wall. OsCCR1, the first committed enzyme of the lignin branch biosynthetic pathway, was identified as interacting with OsRac1 (Kawasaki et al. 2006). Expression of OsCCR1 was induced during immune responses, and the interaction of

OsCCR1 with OsRac1 led to the enzymatic activation of OsCCR1. Thus, it is likely that OsRac1 stimulates the production of monolignols during immune responses by activation of OsCCR1. As mentioned above, OsRac1 also regulates ROS production through interaction with the N-terminal regulatory domain of OsRbohB. Thus, OsRac1 seems to have a dual function in lignin biosynthesis to control synthesis and polymerization of monolignols through regulation of OsCCR1 and NADPH oxidase, respectively (Kawasaki et al. 2006).

Heterotrimeric G proteins consist of α, β, and γ subunits, which are involved in a variety of cellular responses including immunity and development (Urano and Jones 2014). The loss-of-function mutation of Gα subunit (*dwarf1*) reduces PTI and disease resistance to rice blast (Suharsono et al. 2002). OsRac1 functions downstream of Gα in immune signaling. Silencing of *OsRac1* and the *dwarf1* mutation reduce the protein levels and activity of OsMPK6, suggesting a connection among OsRac1, Gα, and the MAPK cascade. In addition, the scaffold protein OsRACK1 was identified as an interactor with OsRac1 and shown to regulate rice immunity in cooperation with OsRac1 (Nakashima et al. 2008). Recently, *Arabidopsis* RACK1 was reported to be a MAPK scaffold protein that connects heterotrimeric G protein with the MAPK cascade (Cheng et al. 2015). Therefore, it is possible that OsRACK1 functions as a scaffold protein for the rice immune network connecting OsRac1, Gα, MAPK, and NADPH oxidase.

ETI is often accompanied by hypersensitive cell death. To understand the molecular mechanism of cell death, rice mutants with lesion-mimic phenotypes in leaves have been utilized (Takahashi et al. 1999). *spotted leaf 11* (*spl11*) is a lesion-mimic mutant, which is caused by a loss-of-function mutation of rice U-box-type E3 ubiquitin ligase (Zeng et al. 2004). SPL11 regulates resistance to *M. oryzae* and *Xoo*. Recently, SPIN6, a Rho GTPase-activating protein (RhoGAP), was identified as a substrate of SPL11 (Liu et al. 2015). In fact, SPL11 ubiquitinates SPIN6 in vitro and degrades SPIN6 in vivo via the 26S proteasome-dependent pathway (Liu et al. 2015). A knockout mutation of *SPIN6* enhanced resistance to *M. grisea*, indicating that SPIN6 is a negative regulator of immunity. Because SPIN6 possesses GAP activity toward OsRac1, it is likely that degradation of SPIN6 by SPL11 upregulates OsRac1-mediated immune responses.

Sekiguchi lesion (*sl*)/*spotted leaf 1* (*spl1*) exhibits unique orange-colored lesions that are induced by infection with *M. oryzae*. The *sl*/*spl1* plants accumulate high levels of tryptamine dependent upon lesion formation (Ueno et al. 2003). The *SL*/*SPL1* gene encodes CYP71P1, cytochrome P450 monooxygenase. SL/SPL1 possesses tryptamine 5-hydroxylase enzyme activity that catalyzes the conversion of tryptamine to serotonin (Fujiwara et al. 2010). Expression of *SL*/*SPL1* gene was induced by treatment with PAMPs and infection with *M. oryzae*, indicating the involvement of serotonin in plant immunity. In fact, exogenous application of serotonin induced the expression of defense genes in OsRac1- and Gα-dependent manner.

18.3 WRKY45-Mediated Immunity

SAR is mediated by the SA signaling pathway. In rice, the SA pathway branches into two different pathways regulated by a WRKY-type transcription factor WRKY45 or a transcriptional cofactor OsNPR1 (Shimono et al. 2007). WRKY45 plays important roles as a transcriptional activator of immune-related genes in resistance to *M. oryzae* and *Xoo* induced by a defense activator benzothiadiazole (BTH) (Shimono et al. 2007). In fact, overexpression of *WRKY45* strongly enhanced resistance to both pathogens (Shimono et al. 2012). Therefore, WRKY45 was utilized as a suitable tool for the development of disease-resistant rice (Goto et al. 2016). Recent investigations also demonstrated that WRKY45 participates in resistance to the root hemiparasite witchweed *Striga hermonthica* (Mutuku et al. 2015).

Panicle blast 1 (*Pb1*) encodes a NB-LRR protein lacking the P loop and the other motifs conserved in typical NLRs, suggesting that Pb1 may not function as a pathogen recognition receptor. Pb1 was identified as an *R* gene that confers non-race-specific, durable, and quantitative resistance to rice panicle blast. Pb1 positively regulated the WRKY45 accumulation by direct interaction with WRKY45 (Inoue et al. 2013), which is considered to be a key step in Pb1-mediated blast resistance.

18.4 Conclusion

Rice blast and bacterial blight cause the most damaging diseases in rice worldwide. The most efficient strategy to prevent these diseases is to develop rice varieties with durable and broad-spectrum resistance. Understanding the molecular mechanism of rice immune responses provided powerful tools such as WRKY45, OsRac1, and the RLCK family for development of disease resistance plants. However, the molecular mechanisms of how plants recognize pathogens and induce immune responses are not fully understood. Isolation of new *R* genes and the identification of new immune factors are required in order to fully understand rice immunity.

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Chapter 19
Interaction of Rice and *Xanthomonas* TAL Effectors

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Abstract Bacterial blight of rice, caused by the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo) in rice, represents one of the most well-studied crop diseases and is also well-known as a model for studying host/microbe interaction. TALEs (transcription activator-like effectors), as a group of pathogenesis factors and once translocated into the host cells from pathogen, recognize and activate host genes to condition disease susceptibility and also trigger host resistance responses dependent on the nature of target genes in plants. TALEs and their target genes have become the foci of the molecular battles between Xoo and rice. The continuing battles have led to incredibly diverse virulence mechanisms in pathogen and counteracting defense mechanisms in rice. Extensive efforts have been made to understand the TALE biology, identify host target genes, and elucidate their interaction and resulting physiological relevance to rice blight and other crop diseases. This review aims to summarize how much we have learned about TALEs and their role in bacterial blight of rice, as well as associated susceptibility and resistance genes in the host. The review also intends to provide a prospect of engineering genetic resistance by applying precise genome editing of TALE-associated target genes in rice.

Keywords TALE · Rice · *Oryza* · *Xanthomonas* · Bacterial blight · Resistance · Susceptibility · Immunity · SWEET gene

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19.1 Introduction

Rice (*Oryza sativa* L.), an important stable crop that provides food for more than half of the world's population, suffers a variety of biotic and abiotic stresses, some of which cause severe loss to crop production and affect global food security (Oerke 2006). During the cropping season, rice diseases occur at different stages. In particular, bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), is one of the most devastating rice diseases (Mew et al. 1993). Bacterial blight of rice is a vascular disease, starting with the pathogen infection of rice through entering the wound and hydathodes of plant leaves, multiplying and colonizing the vascular bundle cells, and eventually resulting in gray to white opaque necrotic lesion, so-called leaf blight (Niño-Liu et al. 2006). There are no effective measures to chemically control this disease in the field; the most effective and economically friendly way to manage the disease is through genetic resistance that is conferred by dominant or recessive disease resistance genes, the collectively so-called R genes that have been recurrently bred into the rice cultivars of interest (Mew 1987). However, newly emerging virulent, pathogenic isolates or races of Xoo can overcome the integrated resistance gene or genes rapidly and render the time-consuming breeding efforts a failure (McDonald and Linde 2002). Therefore, a better understanding of the virulence mechanisms of pathogen and host response to the infection as well as coupling with the advent of biotechnologies and advanced breeding programs will provide promise to effectively control this disease.

The blight disease of rice, like most of other plant diseases, is the outcome of molecular interaction between the host rice and pathogen Xoo. One such interaction is bridged through a type III secretion system (T3SS) of bacterial origin which translocates the bacterial effector proteins into host cells (White and Yang 2009). The effectors function per se to manipulate the host transcriptional and physiological processes, which condition a state of disease susceptibility in host, while host counteracts the effectors by involving changes in target genes and generation of resistance genes to prevent disease from occurring. In turn, pathogen has evolved new mechanisms to adapt the host resistance, leading to newly emerging pathogen isolates. This coadaptation and arms race between rice and Xoo also fit a broad aspect of host/microbe interaction characteristic of a "zigzag" model (Jones and Dangl 2006).

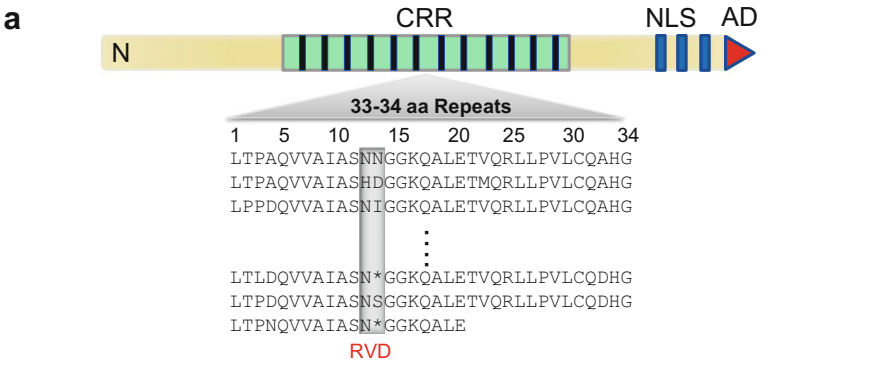
Genes encoding transcription activator-like effectors (TALEs) are present in every sequenced Xoo genome in a large, varying number, ranging from low 9 in AXO1947 to high 19 in PXO99 (Bogdanove et al. 2011; Booher et al. 2015; Huguet-Tapia et al. 2016; Ochiai et al. 2005; Quibod et al. 2016; Salzberg et al. 2008; Yu et al. 2015). In contrast to forming a family of closely related TALEs, other T3SS effectors, so-called non-TAL effectors, are diverse and amount to about 20 in individual Xoo strains (www.xanthomonas.org). Those T3SS effectors collectively play important roles in determining the pathogenesis (resistance and susceptibility) of individual Xoo strains in interacting with their host rice plants carrying diverse genetic background (White and Yang 2009). Additionally,

environmental factors (e.g., humidity and temperature) also influence host resistance (or susceptibility) and eventually the severity of symptom and yield loss of the crop. For instance, unlike in most cases where higher temperature comprises effectiveness of disease resistance genes, the rice R gene *Xa7* becomes more effective in resistance against Xoo strains harboring the cognate avirulence TALE gene *AvrXa7* (Webb et al. 2010).

Serendipitously, bacterial blight of rice has become an excellent model for studying host/microbe interaction due to its genetic amenability, richness of genetic and molecular resource, and a rich history of extensive research endeavor (Niño-Liu et al. 2006). Breakthroughs have been made in identifying key virulence factors, including TALEs that Xoo uses to hijack the host's biological processes to facilitate the infection and disease development, and in discovering host counteracting strategies that make plants resistant. Discovery derived from this rice/Xoo system has spawned development of biotechnological platforms (e.g., TALEN genome editing) and testing strategies for resistance against this particularly devastating disease and maybe applicable to other crop diseases such as citrus canker and cotton blight wherein TALEs also play an important role in pathogenesis. Better understanding of the biological process of disease has facilitated the forming of new and effective disease control strategies with the advent of new biotechnologies. For example, the newly emerging genome editing technologies provide promise for precisely changing the host targets utilized by the pathogen virulence factors or enhancing the resistance process used by the host. The aims of this review article are to summarize the progress that has been made in the past decades in the rice/Xoo interaction mediated by TALEs and to highlight the significance of combining a basic understanding of disease biology with the application of biotech tools to effectively control crop diseases.

19.2 Unique Structural and Functional Domains of TALEs

TALEs have been found to exist in the bacteria of *Xanthomonas* and *Ralstonia solanacearum*, and they form the largest family of T3SS effector proteins. Hundreds of TALE homolog sequences are available at the NCBI (www.ncbi.nlm.nih.gov), where they are highly conserved at the nucleotide and amino acid levels. Each TALE possesses an N-terminal domain with an embedded type III secretion signal, central repetitive region of nearly identical repeats of 33–34 amino acids (CRR), and a C-terminal region with functional nuclear localization signals (NLSs) and a transcription activation domain (AD) (Boch et al. 2010) (Fig. 19.1a). TALEs are DNA-binding proteins that recognize and bind to specific sequences of target genes, namely, the effector binding elements (EBEs), in the host genome. They recognize the DNA sequence of the target following a TALE code that involves (1) one repeat of TALE corresponding to one nucleotide of EBE and (2) one type of repeat preferentially binding to one type of four nucleotides. The identity of each repeat is largely dictated by the composition of two amino acid residues at the positions



b

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
AvrBs3	HD	NG	NS	NG	NI	NI	NI	HD	HD	NG	NS	NS	HD	HD	HD	NG	HD	NG											
PthXo1	NN	HD	NI	HG	HD	NG	N*	HD	HD	NI	NG	NG	NI	HD	NG	NN	NG	NI	NI	NI	NI	N*	ND	N*					
PthXo2	NI	HG	NI	NN	NN	NI	NN	HD	NI	HD	NS	NS	NS	HD	NN	HD	NG	HD	HD	HD	NG	NG							
PthXo3	NI	HG	NI	HG	NI	NI	NI	HD	NN	HD	HD	HD	NG	HD	N*	NI	HD	HD	NN	NS	NI	NN	NN	NG	NN	HD	N*	NS	N*
TalC	NS	NG	NS	HD	NI	NG	NN	NG	HD	NI	NN	N*	NI	NN	HD	NG	NI	NN	N*	HD	NN	NG							
Tal5	NN	HD	NN	HD	NG	HD	HD	NG	HD	NI	NI	NN	HD	HD	N*	NG	HD	NI											
PthXo6	NI	HG	N	INN	NN	NN	NN	NN	HD	NI	HD	HG	HD	NI	N*	NS	NI	NI	HG	HD	NS	NS	NG						
PthXo7	NG	NG	NI	NI	N*	NN	HD	HD	N*	NI	NI	NI	HG	HD	HG	NN	NS	NN	HD	HD	NG	NG							
AvrXa7	NI	HG	NI	NI	NS	HD	NN	HD	HD	HD	NS	N*	N*	HD	HD	NS	NS	NN	NN	NI	NG	NN	NI	N*	NS	N*			
AvrXa10	NI	HG	NI	HG	NI	NI	NN	HD	NI	HD	NN	HG	NS	NG	HD	N*													
AvrXa27	NI	NN	N*	NG	NS	NN	NN	NN	N	INN	NI	N*	HD	HD	NI	NG	NG												
AvrXa23	HD	HD	NN	NN	NG	NG	HD	NS	HG	HD	NG	N*	HD	HD	HD	N*	NN	NI	NN	HD	HI	ND	HD	HG	NN	HG	NG		

Fig. 19.1 TALEs are structurally unique. (**a**). Schematic structure of TALE. TALE consists of three typical regions: N-terminus, central repetitive region (*CRR*), and C-terminus with nuclear localization signal (*NLS*) and transcription activation domain (*AD*). The CRR contains a varying number of 34-amino acid repeats with the two residues at the 12th and 13th positions defined as RVDs to represent individual repeats. (**b**). Representative and important TALEs that play important roles in the pathogenesis of *Xanthomonas* bacteria. The CRRs of individual TALEs are presented as the RVDs. A single letter is used for each amino acid, and asterisk (*) represents a missing amino acid residue at position 13

12 and 13, so named RVD (repeat variable di-residues), which are highly variable in relation to the amino acid residues at other positions (Boch et al. 2009; Moscou and Bogdanove 2009). For instance, the predominant four repeats represented by HD, NI, NG, and NN match to nucleotides C, A, T, and G, respectively. The EBE sites usually are located within the promoter regions of the target genes. After binding to EBE, the TAL effector transcriptionally activates the host target gene, leading to disease susceptibility of the host or triggering host resistance against the otherwise virulent bacteria based on the nature of the host target gene. Therefore, a combination of number of repeats and compositions of RVDs, and the polymorphism of repeat domains, govern the specificity of TALEs for their EBEs and consequentially functionality of each TALE corresponding to the genetic background of the host plants (Bogdanove et al. 2010).

The N-terminal T3SS secretion signal is required for translocation of TALE into host cells and is therefore indispensable for function of TALE (Buttner and Bonas 2002; Szurek et al. 2002). The C-terminal NLSs are functional for directing TALEs to the nuclei of eukaryotic cells and are also required for virulence and avirulence activities of examined TALEs, including TALEs involved in bacterial blight of rice (PthA, AvrBs3, AvrXa7, AvrXa27) (Gu et al. 2005; Van den Ackerveken et al. 1996; Yang et al. 2000; Yang and Gabriel 1995). Additionally, a short sequence of acidic residues at the end of the C-terminus of TALEs is characteristic of eukaryotic transcription activation domains (Szurek et al. 2001; Zhu et al. 1998, 1999). Similar to NLSs, the activation domains of the TALEs AvrXa10 and AvrXa27 are required for their ability to trigger resistance responses in *Xa10*- and *Xa27*-containing rice varieties, respectively, while that of AvrXa7 is required for its ability to trigger resistance in *Xa7* plants and condition a state of disease in otherwise susceptible rice (Gu et al. 2005; Yang et al. 2000; Zhu et al. 1999).

19.3 TALEs as Virulence Factors Condition a State of Host Susceptibility

The virulence function of TALEs was first reported in citrus canker pathogen *Xanthomonas citri* that carried PthA (pathogenicity A) based on the finding that its presence in the strain 3213 was associated with hypertrophy symptoms at infection sites in citrus leaves (Swarup et al. 1991). The rupture of the canker-like lesions was speculated to promote the release and spread of bacterial cells, providing ecological fitness to the pathogen; deletion of the *pthA* gene deprived the pathogen of the ability to cause canker-like lesion (Swarup et al. 1991, 1992). More PthA-like TALEs (e.g., PthA4, PthC, PthAW, and PthA*) of virulence from different strains of *X. citri* have been identified that cause canker-like lesions in citrus (Al-Saadi et al. 2007). Similarly, AvrBs3, initially identified for its ability to trigger resistance responses in *Bs3*-containing pepper plants and found to exist in certain isolates of *X. campestris* pv. *vesicatoria*, induces host cell hypertrophy in susceptible pepper plants lacking *Bs3* and provides benefits to bacterial virulence in the field condition (Marois et al. 2002; Wichmann and Bergelson 2004). Some pathogenic strains of *Xanthomonas* bacteria in cassava and cotton blight diseases also benefit from TALE genes for increased virulence. All sequenced *X. axonopodis* pv. *manihotis* (Xam) strains possess TALE genes (Bart et al. 2012); it has been found that several TALEs ($TALE1_{Xam,}$ $TAL14_{Xam668}$, $TAL20_{Xam668}$) contribute to the strain virulence (ability to multiply and move systemically through the vascular system) in causing cassava bacterial blight (Castiblanco et al. 2013; Cohn et al. 2014, 2016). In cotton blight, caused by *X. campestris* pv. *malvacearum*, Avrb6 enhances disease symptoms of water-soaking and necrosis of the infected tissue and increases the bacterial release from the infected sites in susceptible cotton plants (Yang et al. 1994, 1996).

Remarkably, pathogenic *X. oryzae* pv. *oryzae* depends on critical TALEs to condition a state of disease susceptibility including *in planta* bacterial growth and spread of bacterial cells for colonization of vascular tissue in susceptible rice (Streubel et al. 2013; Sugio et al. 2007; Vera Cruz et al. 2000; Yang and White 2004; Yang et al. 2000; Yu et al. 2011). All sequenced or examined Xoo strains contain multiple copies of TALE sequences which are diverse in terms of their amount of the central repeats and composition of RVDs. The TALE sequences are predicted to express intact (or full-length) TALEs or variants of truncated TALEs (Grau et al. 2016). Six naturally occurring TALEs (AvrXa7, PthXo1, PthXo2, PthXo3, TalC, and Tal5, so-called major TALEs) have been found to be the essential virulence factors of Xoo in causing blight disease. When the major TALE genes were inactivated, the resultant mutant Xoo strains acted like non-pathogenic bacteria, lacking the ability to spread and colonize the vascular tissue of infected rice leaves, a unique feature for rice/Xoo pathosystem (Bai et al. 2000; Yang and White 2004; Yu et al. 2011). In addition, some other TALEs (e.g., PthXo6, PthXo7) moderately contribute to virulence so that some Xoo strains cause blight in susceptible rice (Bai et al. 2000; Sugio et al. 2007). Those TALEs are mainly distinguishable by the number of central repeats and composition of the RVDs (Fig. 19.1b). Their roles in virulence contribution also depend on the identity of their host target genes (see below).

Surprisingly, there are two classes of TALE variants that function as the virulence factors of Xoo to interfere with the resistance in rice that contains the R gene *Xa1* that encodes an NLR (nucleotide-binding domain, leucine-rich repeat) protein and recognizes most, if not all, full-length TALEs as its cognate avirulence effectors (Ji et al. 2016; Read et al. 2016). Those TALE variants were previously annotated as pseudogenes due to the mutations of premature stop codons or large deletions at their 3′ ends of coding sequences but were found to be expressed as the C-terminally truncated proteins, so named iTALEs (interfering TALEs). As the prototypes characterized from PXO99^{A}, *Tal3a* with a premature stop codon encodes a TALE with a C-terminal truncation of 103 amino acids (the last NLS and AD deleted), while *Tal3b* carries a large deletion within its 3′ end results in a predicted protein with 229 amino acids deleted (knockout of three NLSs and AD) and 10 amino acids added (including a single, new NSL) due to a frame shift. Both genes contain two identical deletions (129 and 45 bp) within their 5′ regions compared to the intact TALE genes. Tal3a and Tal3b contain identical amino termini, distinguishable CRRs, and distinct C-termini. Deletion of the clustered *Tal3a* and *Tal3b* genes resulted in a mutant of PXO99^{A} that triggered typical HR (hypersensitive reaction)-associated resistance in *Xa1*-containing rice. Furthermore, such 2 types of TALE variants are prevalent among 29 out 36 worldwide Xoo strains and all 9 sequenced *X. oryzae* pv. *oryzicola* (Xoc) strains (Ji et al. 2016). In another study, one such TALE variant, Tal2h named as truncTALE, from Xoc strain BLS256, was found to suppress resistance conferred by the *Xo1* locus (an unknown but most likely NLR R gene) in the heirloom rice variety Carolina Gold (Read et al. 2016). The molecular mechanisms for *Xa1*-mediated recognition

and activation of resistance to TALEs as well as how iTALEs or truncTALEs to interfere with such *Xa1* resistance remain to be determined.

19.4 TALEs as Avirulence Factors Elicit Resistance Responses in Rice Against Xoo Infection

Some TALE genes, like all other microbial avirulence genes, were identified as the genetic determinants for eliciting resistance responses in host plants that contain the cognate resistance (R) gene. The resistance responses include activation of defense genes and hypersensitive reaction (HR). AvrBs3, the prototypic member of TALEs, was cloned from *X. campestris* pv. *vesicatoria* on its ability to trigger resistance responses in the R gene *Bs3*-containing pepper (Bonas et al. 1991; Minsavage et al. 1990). TALE genes *avrXa10* and *avrXa7* were later cloned from the Xoo strain PXO86 based on their homology to *avrBs3* and their abilities to elicit resistance responses in rice varieties containing the R gene *Xa10* and *Xa7*, respectively (Hopkins et al. 1992). *avrXa7,* characterized in the Korean, Japanese, and Philippine Xoo strains, is an effector gene with a dual function of virulence and avirulence depending on the genetic context of the host rice (Ochiai et al. 2000; Yang and White 2004). Similarly, TALEs of avirulence, AvrXa27 and AvrXa23, were cloned from PXO99^{A} based on their relationships with the respective R genes *Xa27* and *Xa23* (Gu et al. 2005; Wang et al. 2014). Those four TALEs are also distinguishable by their number of central repeats and composition of RVDs (Fig. 19.1b), which both collectively determine the resistance specificity of the pathogen races and host cultivars. The resistance response is mediated in a gene-for-gene manner, i.e., one avirulence gene corresponds to one cognate R gene in Xoo and rice, respectively. On the other hand, most, if not all, TALEs function as resistance elicitors (avirulence factors) in rice that possess *Xa1* or *Xa1*-like NLR R gene(s). The specificity of race/cultivar is not determined by the identity of TALEs but instead by the presence of iTALEs or truncTALEs (Ji et al. 2016; Read et al. 2016; Triplett et al. 2016).

19.5 TAL Effectors Induce Disease Susceptibility (S) Genes for Xoo Strain Virulence

Typical TALEs contain the characteristic eukaryotic transcription activator domains (NLS and AD), which tempt the initial hypothesis of host gene activation for the function of TALEs. The early work revealed the genes differentially activated by AvrBs3 in pepper using the cDNA-amplified fragment length polymorphism technique (Marois et al. 2002). The association of host gene induction and virulence functionality of pathogen TALEs came with the identification of

Os8N3 (a member of *MtN3* gene family) induced by its cognate PthXo1 in bacterial blight of rice (Yang et al. 2006). In that work, the rice microarrays were used to identify the differentially expressed genes from rice leaf samples treated with PXO99^{A} and its mutant lacking *pthXo1*. RNAi-mediated gene silencing of *Os8N3* in susceptible rice varieties or natural mutations in the *Os8N3* promoter of rice IRBB13 (a recessive R gene, *xa13*, containing rice) rendered either plant resistant to PXO99^{A} that relied on *pthXo1* for virulence (Yang et al. 2006). The *MtN3* gene family including *Os8N3* was later found to encode a family of sugar transporters, so-called SWEETs, and *Os8N3* or *Xa13* (a dominant allele of *xa13*) was, therefore, renamed *OsSWEET11* (Chen et al. 2010). Other SWEETs were also found to be specifically induced by other major TALEs of virulence. For example, *OsSWEET13* is specifically induced by PthXo2 (Zhou et al. 2015); *OsSWEE14* is induced by multiple TALEs, AvrXa7, PthXo3, TalC, and Tal5 (Antony et al. 2010; Streubel et al. 2013; Yu et al. 2011) (Fig. 19.2). Other two clade-III SWEETs in rice have been shown to also be the susceptibility genes to Xoo when induced with Xoo strains containing the artificial or designer TALEs (Li et al. 2013; Streubel et al. 2013).

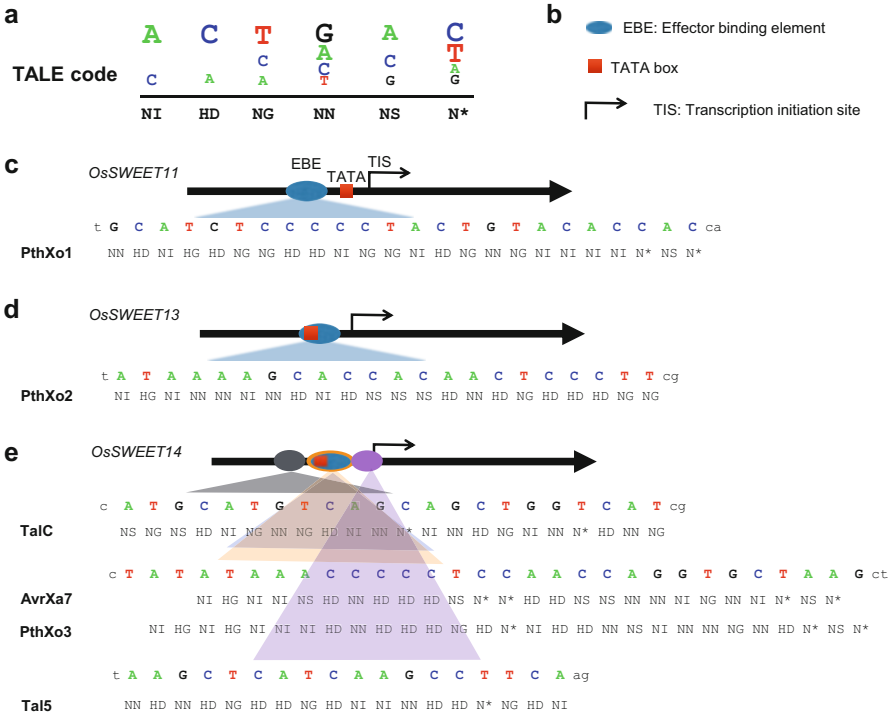


Fig. 19.2 Major TALEs and their SWEET targets in rice blight. (**a**). The TALE code in terms of nucleotides of target DNA and corresponding TALE repeats as illustrated in RVDs. (**b**). Symbols used for important components in the promoters of SWEETs. (**c**–**e**). Schematic gene structures of three SWEETs and their associated, cognate TALEs (presented as RVDs)

The relationship between TALEs and SWEET genes for disease susceptibility has been expanded from the originally described rice/Xoo system to other plant diseases such as cassava/*X. axonopodis* pv. *manihotis* (Cohn et al. 2014) and cotton/*X. citri* pv. *malvacearum* (Cox et al. 2017). The prevailing hypothesis for SWEETs to condition disease susceptibility is that elevated membrane-bound SWEETs render the sugar, especially sucrose, the direct product of photosynthesis in plant, leaking into the extracellular space wherein bacteria use carbohydrate to grow (Chen et al. 2010). Alternatively, leaking sugar out of plant cells depletes the cells of nutrient, and starvation of host cells results in compromised host immunity. Either hypothesis needs to be experimentally validated. TALEs of moderate virulence in Xoo also induce the cognate host target genes of non-SWEET to condition disease susceptibility. For example, PthXo6 and PthXo7 from $PXO99^{A}$ activate two host transcription factor genes, respectively, one for bZIP transcription factor and another for the general transcription factor TFIIA small subunit in chromosome 1 in rice (Sugio et al. 2007). Additionally, TALE of *X. citri* spp. *citri* plays an important role in citrus canker by targeting the host canker susceptibility gene *lateral organ boundaries CsLOB1*, also a transcription factor (Hu et al. 2014).

19.6 Host Resistance in Response to TALEs

To counteract the virulence strategy of pathogens that targets host genes of physiological importance through binding to their promoters and transcriptionally activating the gene expression, plants have evolved a "trapping mechanism" by luring some TALEs to the promoter of defense genes or R genes that have stronger defense activity than classic defense genes (e.g., pathogenesis-related *PR* genes) and are functionally equivalent to the dominant R genes. Those types of R genes are also called "executor" R genes (Bogdanove et al. 2010). The promoter element to which TALEs bind is also called EBE, the effector binding element. The first such case was identified for the pair of *Xa27* and *avrXa27* wherein rice variety IRBB27 harboring *Xa27* recognizes the AvrXa27-secreting Xoo strains through trapping AvrXa27 to the promoter of *Xa27* for its activation and subsequent resistance including hypersensitive responses at the infection sites (Gu et al. 2005). The nature of resistance/susceptibility of the variety and Xoo race is determined by the polymorphism where AvrXa27 could or could not match the EBE of *Xa27* or *xa27*. Similarly, resistance genes *Xa10* and *Xa23*, the closely related R gene family members, specifically lure the cognate TALEs AvrXa10 and AvrXa23 to their promoter EBEs for gene activation and resulting HR-associated resistance in specific varieties (Tian et al. 2014; Wang et al. 2014). Such pairing of TALEs of avirulence and executor R genes also exists beyond rice blight. The pepper R gene *Bs3* (encoding a flavin monooxygenase) traps AvrBs3 for manifestation of resistance against *Xanthomonas campestris* pv. *vesicatoria* harboring the elicitor AvrBs3; similarly, *Bs4C* (encoding a unique R protein) again in pepper was found to be induced by TALE AvrBs4 for disease resistance; in both cases, the

matchings between the EBEs and RVDs are required for gene induction and resistance, and polymorphisms between the resistant and susceptible alleles are primarily attributable to the outcome of diseases (Römer et al. 2007; Strauss et al. 2012).

Rice uses another resistance strategy to prevent its disease susceptibility genes from being induced by TALEs of virulence. Naturally occurring alleles with polymorphisms in EBE of *OsSWEET11* (*Os8N3* or *Xa13*) for PthXo1 are collectively named *xa13*; alterations of 1-bp substitution in rice variety BJ1 and 243-bp insertion in IRBB13 relative to the susceptible allele in IR24 are attributable to the mismatching between RVDs of PthXo1 and its cognate EBE and abolishment of *xa13* induction by PthXo1 (Chu et al. 2006; Yang et al. 2006; Yuan et al. 2009). *xa13* represents alleles carrying the promoter mutations instead of knockout mutations. It, presumably like *Xa13*, encodes a SWEET protein competent for normal physiological function of sucrose transport in the absence of bacterial infection. Another recessive R gene *xa25* was found to be allelic to the bacterial blight susceptibility gene *OsSWEET13* and was implicated as a host target of a major TALE of the Xoo strain PXO339 (Liu et al. 2011). It has been identified and experimentally validated later that *xa25* is a nonresponsive allele of *OsSWEET13* to another TALE, PthXo2, and that the EBE polymorphism in *xa25* and *OsSWEET13* determines the compatibility between respective rice varieties and Xoo strains that depend on PthXo2 alone for virulence (Zhou et al. 2015). The third recessive R gene due to loss of susceptibility to the Xoo TALEs AvrXa7 and Tal5 is *xa41(t)*. The *OsSWEET14* gene in *Oryza barthii* and *O. glaberrima* contains an 18-bp deletion just within the EBE for AvrXa7 and Tal5 relative to *OsSWEET14* in *Oryza sativa*. The natural polymorphism or mutation in *xa41(t)* is responsible for non-response of *OsSWEET14* to AvrXa7 and Tal5 for induction and, thus, responsible for the resistance to the specific Xoo strains (Hutin et al. 2015).

The third strategy deployed by rice for resistance against Xoo pathogens is to also interfere with S gene induction by TALEs not through alteration in EBEs of the S SWEET genes but instead through encoding a variant of general transcription factor TFIIA small subunit, the gene product of the recessive R gene *xa5*. *xa5*, being located on rice chromosome 5, encodes a protein with a valine to glutamic acid change (V39E) in TFIIAγ5 (or Xa5) (Iyer and McCouch 2004; Jiang et al. 2006). TFIIAγ5 is part of the transcription pre-initiation complex (Mediator) that is globally conserved across eukaryotic organisms (Allen and Taatjes 2015). Resistance to bacterial blight conferred by *xa5* in IRBB5 line is broad spectrum against many strains of Xoo (Garris et al. 2003; Iyer and McCouch 2004; Mishra et al. 2013). It has been recently found that *xa5* compromises the gene induction of *OsSWEET13* and *OsSWEET14* by their cognate TALEs PthXo2, PthXo3, and AvrXa7 (Huang et al. 2016). It has been hypothesized that reduced levels of OsSWEET13 and OsSWEET14 in the infected rice tissue are not sufficient enough to support bacterial multiplication. However, *xa5* is not effective to resist to Xoo strains that contain PthXo1 for targeting *OsSWEET11* for virulence. This is probably because the *OsSWEET11* transcript as induced by PthXo1 in *xa5* plants is exceptionally high, a level above the threshold for bacteria to be pathogenic (Huang et al. 2016). It has also been found that induction of *Xa27* by TALE AvrXa27 is

compromised and the resulting resistance is diminished in the *xa5* background in bacterial blight (Gu et al. 2009). The hypothesis that TALEs directly interact with the plant Mediator for gene induction of S genes has been tested recently, and direct interaction of TFIIAγ5 with several TALEs has been demonstrated (Yuan et al. 2016).

19.7 Concluding Remarks

Through decades of research endeavor by scientists who had and have been working on bacterial blight of rice, significant progresses or breakthroughs have been made in the understanding of the biology of this disease. Achievements include revealing the key components of T3SS effectors (especially TALEs) and host target genes, and elucidating their interactions at the molecular level, which all play roles in bacterial infection, spread, and colonization in vascular tissue as well as host responses. In a simple model as illustrated in Fig. 19.3, the disease process starts with contact of Xoo with the plant cells and secretion and translocation of TALEs through T3SS into cytoplasmic space (scenario 1); TALEs go to nuclei and activate S genes for disease (scenario 2); plants change EBE sequences to avoid gene induction for loss of disease susceptibility (a resistance different from a dominant R gene-mediated resistance) (scenario 3); plants employ *xa5* to reduce S gene induction by certain TALEs (scenario 4); plants also evolve executor R genes to trap TALEs to trigger resistance (scenario 5); and finally plants use the dominant NLR R genes to recognize TALEs and activate resistance independent of gene activation (scenario 6); in return Xoo mutates some TALEs to not only avoid NLR R protein recognition but also actively suppress resistance triggered by their progenitor TALEs (scenario 7). It is worthy to note that the genetically diverse Xoo populations produce several major TALEs capable of targeting multiple S SWEETs to overcome the usually single recessive resistance gene derived from the S SWEETs in rice.

The TALE targets (e.g., SWEETs) present a potential target for genetic manipulation of rice genome through biotechnologies for engineering broad resistance. Given the fact that the naturally occurring polymorphisms of S genes do not affect the normal physiological function of target genes, biotechnologies for such gene editing provide promise to mimic such polymorphisms for improved traits with possibly little negative effect on the overall performance of rice plants. The advanced genome-editing technologies enable precise, targeted genomic changes to specific loci. The editing-enabling technologies include zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), and most recently clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) proteins (Weeks et al., 2016). The first TALEN-edited rice was generated by altering the overlapping EBEs of *OsSWEET14* promoter for AvrXa7 and PthXo3 (Li et al. 2012). Through genetic transformation, TALEN reagents specifically targeting *OsSWEET14* in rice susceptible to AvrXa7-dependent Xoo bacteria were used to generate plants with high mutagenesis frequency. Progeny plants homozygous for

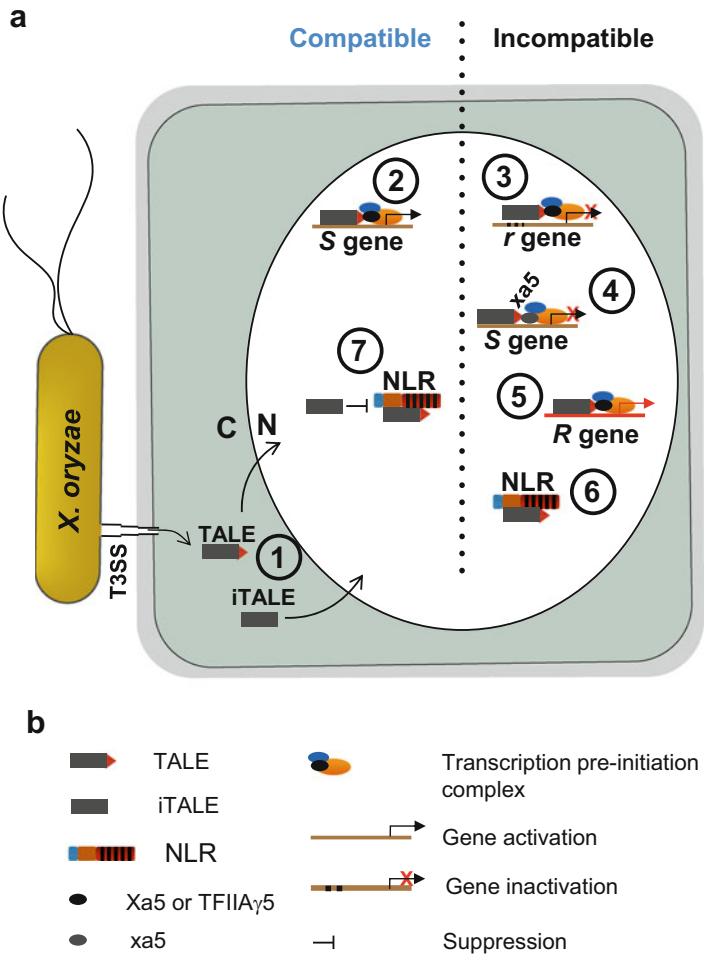


Fig. 19.3 Molecular interactions between TALEs and the cognate rice factors and the resulting bacterial blight disease. (**a**). In scenario 1, pathogen (e.g., Xoo) injects intact TALE and its variant iTALE into the cytoplasm (*C*) of host cell through a type III secretion system (*T3SS*); TALE and iTALE are localized to the cell nucleus (*N*) through their nuclear localization signals. In scenario 2, TALE targets the host disease susceptibility (*S*) gene through binding to its EBE (*effector binding element*) and transcriptionally activating the S gene, leading to a state of disease susceptibility, a compatible interaction between pathogen and host. In scenario 3, mutations (black dots) in EBE of the S gene in certain rice varieties prevent its activation by TALE, resulting in incompatible interaction between such a host and pathogen; the TALE-irresponsive allele of S gene becomes a genetically recessive resistance (*r*) gene. In scenario 4, rice with its *TFIIA* small subunit gene (*TFIIAγ5*) mutated (*xa5*) becomes incompatible to Xoo due to the inefficient activation of S gene by TALE. In scenario 5, rice use executor R gene to trap TALEs to trigger host resistance through gene activation. In scenario 6, rice also uses the NLR R gene to recognize TALEs for resistance independent of R gene activation. Finally, in scenario 7, pathogen uses truncated TALE variants to suppress TALE-triggered, NLR R gene-mediated resistance for compatible interaction between the host and pathogen. (**b**). Symbols used in Fig. 19.3a

the EBE mutations and free of the TALEN transgene were highly resistant to Xoo strains that depended on AvrXa7 to induce *OsSWEET14*-associated rice blight (Li et al. 2012). A similar approach was used to mutate the EBEs in the promoter of *OsSWEET14* targeted by TalC and Tal5, two TALEs prevalent among the African Xoo strains. Plants with respective mutations exhibited strong resistance to TalC- and Tal5-dependent strains that were pathogenic to the otherwise susceptible parental plant (Blanvillain-Baufumé et al. 2017). These studies provide the proof-of-concept successful cases in manipulation of a single SWEET gene for blight resistance. Multiplex genome-editing technologies based on CRISPR/Cas most likely enable simultaneous mutations in a single rice line of all five known EBEs in three S SWEETs (*OsSWEET11*, *13*, and *14*) targeted by all known TALEs. Such rice will be expected to confer the broadest resistance to most of, if not all, Xoo strains that are virulent to the otherwise susceptible parental rice.

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Chapter 20
Marker-Assisted Gene Pyramiding for Durable Resistance to Blast

Shuichi Fukuoka

Abstract Effective control of blast, a devastating fungal disease of rice caused by *Pyricularia oryzae*, is an important breeding objective to increase and stabilize worldwide rice production. Use of quantitative resistance to blast is considered a promising strategy because such resistance is more durable than race-specific qualitative resistance. Quantitative resistance is under complex genetic control, with each contributing gene having a smaller individual effect than those conveying qualitative resistance. This characteristic decreases selection pressure, thereby slowing the counter-evolution of the pathogen, but also makes it difficult for breeders to select the required traits in practical breeding programs. Extensive genetic studies of quantitative resistance during the last two decades have identified several relevant genes and have contributed to our understanding of the genetic control of quantitative resistance. Such studies will accelerate marker-assisted gene pyramiding of resistance alleles to confer strong, non-race-specific, and environmentally stable resistance to blast disease; this achievement would contribute substantially to global food security.

Keywords Quantitative resistance · Partial resistance · Quantitative trait loci · Durable resistance · Marker-assisted selection · Gene pyramiding · Rice · Blast disease

20.1 Introduction

Resistance to blast disease in rice was first described by Sasaki (1923), and many studies identifying resistant loci were conducted from the 1960s to 1980s (for review, see McCouch et al. 1994). After the completion of the genome sequence of rice and blast fungus, infection of rice by blast has been used as a model for the study of plant–pathogen interactions, in addition to its significance in crop

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Table 20.1 Terminologies that refer to two categories of resistance to blast disease[a]

Criteria		
Level of resistance	Qualitative resistance	Quantitative resistance
	Complete resistance	Incomplete (partial) resistance
Response to infection	Resistant infection type (hypersensitive response [HR])	Susceptible infection type (lack of HR)
Mode of inheritance	Major gene resistance (monogenic resistance)	Minor gene resistance (polygenic resistance)
Race-specificity	Race-specific resistance	Non-race-specific resistance
	Vertical resistance[b]	Horizontal resistance[b]
	True resistance[c]	Field resistance[c]

[a]Terminologies refer to two categories of the resistance of certain cultivars, on the basis of several criteria indicated in the column on left. Note that a gene identified for "polygenic" resistance could be dealt with as a Mendelian factor in backcrossed progeny lines (See Sect. 20.3.6). Also, note that a gene identified in a cultivar showing "non-race-specific resistance" might show race-specificity (See Sects. 20.3.3 and 20.3.6)
[b]Van der Plank (1963)
[c]Muller and Haigh (1953)

improvement for the demands of an increasing global population (Dean et al. 2005; International Rice Genome Sequencing Project 2005). Like in other plant–pathogen interactions, two categories of resistance to blast have been described in rice (Ezuka 1972; Parlevliet 1979); these are based on the magnitude of effect on inhibition of the pathogen reproduction, mode of inheritance, and race-specificity (Table 20.1), as outlined below. In this review, we refer to these categories as qualitative and quantitative resistance.

Qualitative resistance is usually conditioned by a single resistance (*R*-) gene that acts in a race-specific manner and is frequently associated with hypersensitivity to the pathogen (Flor 1971). Despite originally having significant resistance against rice blast, the cultivars with qualitative resistance have lost it several years after their release (Bonman et al. 1992; Kiyosawa 1982). This can be explained by rapid increases in the pathogen races that are compatible with the genes for qualitative resistance and has demonstrated the need to enhance resistance through genes for both qualitative and quantitative resistance.

Quantitative resistance is usually incomplete, that is, although it limits the proportion of leaf that is diseased, some disease susceptibility remains. This resistance is considered to be durable, because it is maintained for a long time (Poland et al. 2009). Quantitative resistance is usually under the complicated genetic control of quantitative trait loci (QTLs). In the early twenty-first century, the genetic basis and mechanism of quantitative resistance to blast was not fully understood, especially with regard to host gene structure and race-specific interaction with blast. Therefore, researchers focused on identifying genes for quantitative resistance to understand resistance durability and the differences between qualitative and quantitative resistance.

This chapter describes progress in understanding genetic control of quantitative disease resistance, as well as the use of marker-assisted introgression of multiple

genes into susceptible genetic backgrounds to enhance quantitative resistance as a reliable strategy for conferring durable resistance in rice.

20.2 Detection of Chromosomal Regions Involved in Resistance to Blast

Identification of loci that control resistance to blast is the first step in efforts to improve rice cultivars by incorporating beneficial alleles into superior genetic backgrounds. Genetic analysis using DNA markers allows us to determine chromosomal locations associated with agricultural traits, even those under complex genetic control (Tanksley and Nelson 1996). Generally, genes with a small effect, which confer quantitative resistance, are harder to detect than those with a major effect, which confer qualitative resistance. Thus, the mapping of genes for rice blast resistance initially concentrated on qualitative resistance; more than 100 loci have been identified, and more than 20 of them have been cloned (Ashkani et al. 2016; Koide et al. 2009) (Fig. 20.1). Most of these genes encode proteins with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (NBS–LRR proteins) that interact with pathogen effectors and trigger defense reactions following a gene-for-gene relationship (Bryan et al. 2000; Cesari et al. 2013), except *Pi-d2*, which encodes a receptor-like kinase protein (Chen et al. 2006). There is also evidence for epistatic control by a pair of tightly linked NBS–LRR genes (Ashikawa et al. 2008; Lee et al. 2009; Okuyama et al. 2011). Despite the idea that research on quantitative resistance will uncover novel aspects of plant defense and be used to develop cultivars that are durably resistant to blast, progress for quantitative resistance is slower than that for qualitative resistance because gene effects underlying restriction of lesion area, a typical phenotype of quantitative resistance, are substantially sensitive to environmental factors (Fig. 20.2).

Analyses of quantitative resistance need to be conducted by using either (a) mapping populations from a cross between parents that lack genes for qualitative resistance or (b) subpopulations derived from populations in which plants showing the resistance phenotype were eliminated. Therefore, researchers need to take more care in preparing a mapping population for quantitative resistance than for qualitative resistance. After the first report on DNA marker-based mapping of quantitative resistance (Wang et al. 1994), extensive studies of quantitative resistance have detected more than 350 QTLs, in a total of more than 20 chromosomal regions (Ballini et al. 2008). The observation that these chromosomal regions co-localize with loci for qualitative resistance (Fig. 20.1) supports the hypothesis that many genes for quantitative resistance are weak alleles of *R*-genes (Ballini et al. 2008; Xu et al. 2008).

Because of the low mapping resolution and accuracy of estimates of additive effects of alleles in genetic analyses using primary mapping populations, such as F_2 or recombinant inbred lines (RILs), it has been difficult to delimit the map positions

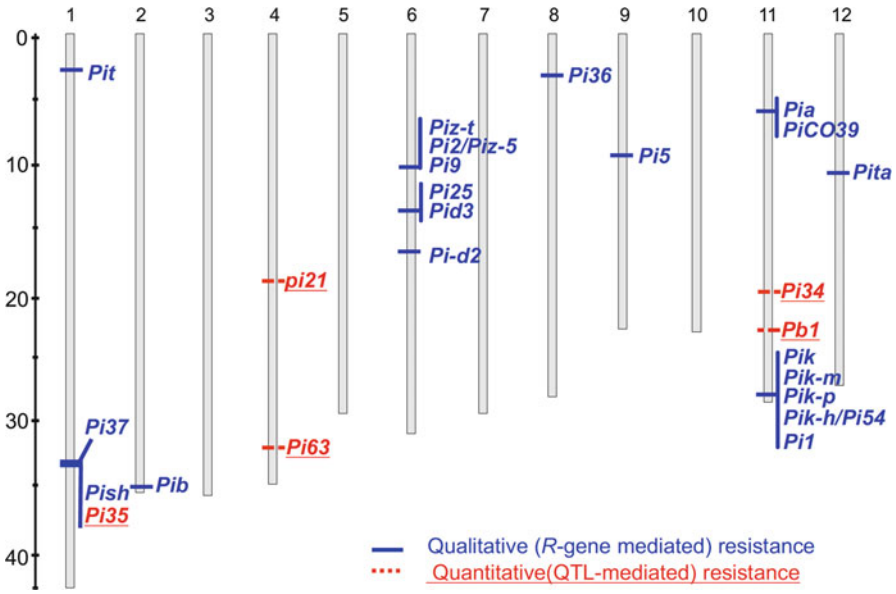

Fig. 20.1 Cloned genes for blast resistance. Genes identified through their naturally occurring variations are indicated. Qualitative (*R*-gene mediated) resistance (blue), quantitative (QTL-mediated) resistance (red underlined). Positions in Mb (IRGSP 1.0) (Kawahara et al. 2013) are indicated on the left side of the map. *Pish* and *Pi35* are allelic to each other (See Sect. 20.3.2). *Pi34* has not been cloned, but characterization of a candidate gene is in progress

of QTLs for blast resistance and to validate their effects. One strategy to solve this problem is to develop backcrossed progeny lines for each QTL by using marker-assisted selection (MAS; Tanksley and Nelson 1996). Because the genetic background of backcrossed lines is more uniform that that of primary mapping populations, their use increases the ability to detect QTLs with small effects (Yamamoto et al. 2009; Yano and Sasaki 1997). Furthermore, reference genome information of rice and re-sequencing information allows us to apply MAS even to crosses between closely related cultivars (Hori et al. 2012; McCouch et al. 2010; Yamamoto et al. 2010).

20.3 Identification and Characterization of Genes for Quantitative Resistance

Fine genetic analysis of quantitative resistance has significant impact on our understanding of plant defense as well as having practical importance for crop improvement. This section describes five genes (*pi21*, *Pi35*, *Pi63*, *Pb1*, and *Pi34*) for quantitative resistance to blast that have been fine mapped or cloned.

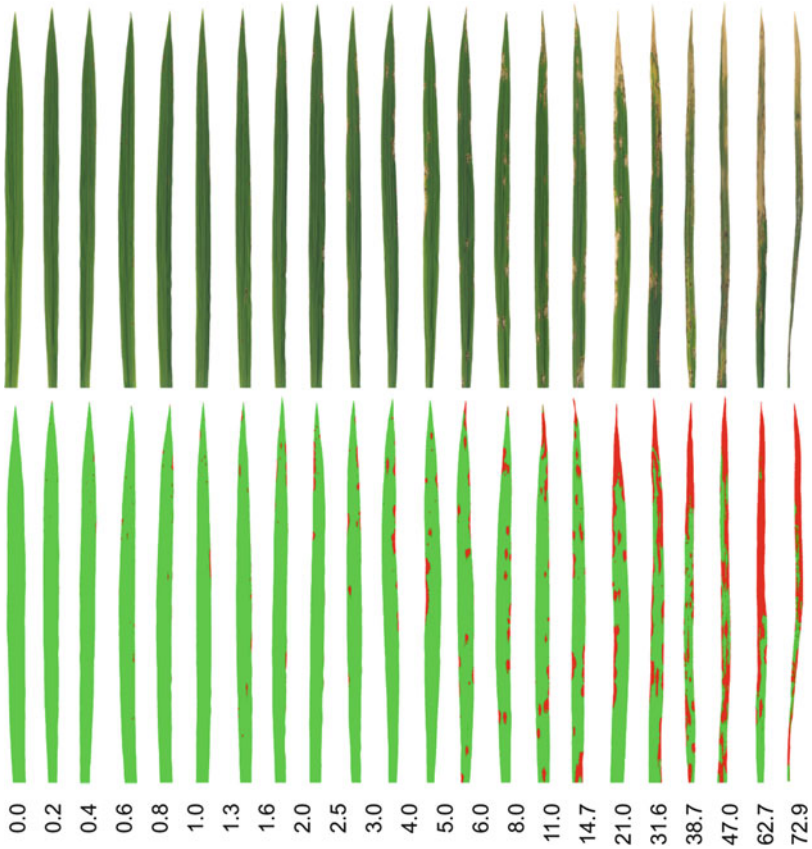

Fig. 20.2 Measurement of quantitative resistance based on lesion area. Plants that differ in the level of quantitative resistance were inoculated with blast fungus, and leaves that differ in disease severity were selected as a reference of lesion area. Leaf images obtained with a digital image scanner (upper). Transformed leaf images showing diseased (red) or non-diseased (green) areas. Lesion area (%) was calculated as the ratio of the infected area to the total leaf area × 100, as indicated below the images

20.3.1 Loss-of-Function Mutation in Gene Encoding a Proline-Rich Protein Confers Non-race-Specific Resistance to Blast

The *Pi21* locus was originally identified in a Japanese upland rice cultivar, "Owarihatamochi," as the QTL with the largest resistance effect in a mapping population derived from a *japonica* irrigated and upland rice cross, whose resistance is recessively inherited (Fukuoka and Okuno 2001). To date, three recessive resistance loci for blast resistance, *Pi21*, *Pi55*(t), and *Pi66*(t), have been reported.

Among these, only *Pi21* has been characterized at the molecular level (Fukuoka et al. 2009; He et al. 2012; Liang et al. 2016); therefore, this section focuses on *Pi21*.

Map-based cloning of *pi21* gene unveiled that this gene encodes a proline-rich protein with a putative heavy-metal-binding domain and putative protein–protein interaction motifs. Comparison of nucleotide variation at the *Pi21* locus among Asian cultivated rice suggests that the resistance allele is found only in *japonica* upland rice cultivars. Unlike other *R*-genes, the resistance allele of *Pi21* does not inhibit pathogen infection; rather it confers moderate resistance by preventing growth of hyphae in the plant cell (Fukuoka et al. 2009). The resistant *pi21* allele is effective against diverse fungal races, so use of *pi21* might not be a strong driving force to change the pathogen population structure. Therefore, *pi21* is considered more durable than other *R*-genes, although durability of *pi21* needs to be proved in cultivars carrying this gene alone under natural field conditions (Fukuoka et al. 2009).

In comparison with the susceptible *Pi21* allele, the resistant *pi21* allele carries two in-frame deletions in regions that carry proline-rich motif sequences involved in protein–protein interactions in multicellular organisms (Ball et al. 2005; Pawson 1995). Cultivars carrying one of the two deletions or two smaller deletions were not resistant. Hence, the two deletions in the resistant *pi21* allele might be optimal for loss of function. Interestingly, among Asian cultivated rice, DNA variations that cause amino acid changes in the product of *Pi21* were only found in the proline-rich motif sequences in the middle part of the gene; those in the C-terminal region and a putative heavy-metal-binding domain in the N-terminal region were free from variations. Such observations imply that the resistance *pi21* allele might maintain certain functions whose loss would be deleterious for plant growth. Lower survival rate of plants whose *Pi21* expression is strongly suppressed by RNAi is consistent with this hypothesis (Fukuoka, unpublished data).

Evidence with plant defense by *Pi21* is still limited, despite this gene being known to have an effect on the interaction between *Arabidopsis* and blast fungus (Nakao et al. 2011). Compared with plants with the susceptible *Pi21* allele, plants carrying a resistant *pi21* allele show higher expression of pathogenesis-related genes at 3–6 h after inoculation with a virulent race. This phenomenon is observed when elicitor solution prepared from fungal spore suspensions is sprayed on the plants, implying that *pi21*-mediated resistance accompanies elicitor-triggered immunity (Fukuoka et al. 2008).

Ethylene induces resistance to a number of diseases when applied before infection, and inhibition of its biosynthesis decreases resistance (van Loon et al. 2006). Rice plants carrying a resistant *pi21* allele are insensitive to pre-inoculation application of an antagonist of ethylene biosynthesis, 2-aminoisobutyric acid, whereas pre-inoculated plants that carry the susceptible *Pi21* allele develop significantly larger lesions than observed in the corresponding untreated with antagonist (Fukuoka et al. 2015). This observation implies that *pi21*-mediated resistance involves ethylene signaling. In contrast to some other defense genes, such as *WRKY45* and *BSR1*, that alter resistance to multiple plant pathogens (Maeda et al. 2016; Shimono

et al. 2007), *pi21* does not affect resistance to a bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* or a different fungal pathogen *Rhizoctonia solani* (Fukuoka et al. 2009). Therefore, the loss of function associated with *pi21* might specifically inhibit hyphal growth of blast pathogen through heavy metal transport and protein–protein interaction. Identification of protein(s) interacting with Pi21 would provide further insight into mechanism of *pi21*-mediated resistance.

20.3.2 Pi35*, an Allele of a Typical* R*-Gene Whose Multiple Functional Polymorphisms Confer Quantitative Resistance to Blast*

Map-based cloning and characterization of the *Pi35* locus has provided important information to clarify the relationship between the genes for qualitative and quantitative resistance. This section mainly focuses on a comprehensive study of allelic variation of this locus (Fukuoka et al. 2014), as well as other studies that explore its involvement in the reaction against pathogen isolates of different origins.

Pi35 is derived from the Japanese breeding line "Hokkai 188" that has maintained consistent resistance in natural field conditions since 1961. Therefore, it has been used as a source of quantitative resistance in the Japanese rice breeding program. Previous studies roughly mapped this gene to chromosome 1 in or near the region containing race-specific qualitative resistance gene loci *Os01g0781700* (*Pi37*) and *Os01g0782100* (*Pish*) (Lin et al. 2007; Nguyen et al. 2006; Takahashi et al. 2010). High-resolution genetic mapping of *Pi35*, using a population derived from a cross between resistant "Hokkai 188" and susceptible "Danghang-Shali," delimited this locus to a 59.2-kb region harboring the above two *R*-gene loci and another two gene loci containing resistance gene analogs (RGAs) with an NBS–LRR domain. Sequence comparison of "Hokkai 188" with "Nipponbare," which carries *Pish* gene, revealed overall similarity, but a 20.1-kb deletion in "Hokkai 188" has resulted in the loss of two gene loci (*Os01g0781200* and *Os01g0781700*). Out of 17 sequence variations between "Nipponbare" and "Hokkai 188" in the *Pi35* locus, only 6 sequence variations being located in *Os01g0782100* showed variation in the deduced amino acid sequence, narrowing down *Os01g0782100* as the candidate for *Pi35* gene, and thus *Pish* and *Pi35* are alleles of the same gene; this finding was confirmed by complementation testing (Fukuoka et al. 2014).

Because the level of *Pi35* expression is the same as that of *Pish* before and after inoculation, variations in the coding region of *Os01g0782100* control the differences in lesion area and level of *pathogenesis-related gene 2* (*PR2*), an indicator of the strength of defense response at the tested time points, between plants carrying *Pi35* and those carrying *Pish* (Fukuoka et al. 2014). At 48 h after inoculation with an avirulent isolate (Kyu77-07A), plants carrying *Pi35* show similar or higher expression of the *PR2* than those carrying *Pish* do, and this expression is higher than in plants carrying *Pish* after inoculation with a virulent isolate (Ina 86–137).

The LRR region of NBS–LRR proteins plays a critical role in determining race-specificity (Michelmore and Meyers 1998; Parker et al. 1997), whereas the NBS domain is generally considered to be involved in signal transduction (Takken et al. 2006; Traut 1994). In an analysis using chimeric gene constructs in plants containing *Pish*, replacing the LRR-encoding region of *Pish* with that of *Pi35* resulted in (a) loss of race-specific resistance to the avirulent isolate and (b) quantitative resistance to the avirulent and virulent isolates. Of the four amino acid residues in the LRR region of Pi35 that differ from those in Pish, substitution of Glu to Asp codon at position 1054 in Pish was the most effective mutation for reducing lesion area, although this was still not sufficient to confer the full effect of Pi35.

The case of *Pi35* indicates that a combination of multiple functional polymorphisms with small individual effects on reduction of lesion area confers a practical level of resistance. As mentioned in the Introduction, qualitative but race-specific resistance is easily overcome by rapid evolution of the fungal pathogen (Bonman et al. 1992; Kiyosawa 1982), and enhancing resistance by combining multiple variations in a gene might confer a more durable form of resistance, owing to decreased selection pressure against the pathogen. Since the frequency of accessions with the most effective mutation in the *Pi35* gene (i.e., the mutation that results in Glu to Asp substitution at position 1054) is low among Asian cultivated rice accessions, this mutation might have arisen recently. *Pi35* confers quantitative resistance with wider spectrum compared with *Pish*, which exhibits race-specificity in Japan; this suggests that *Pi35* might be the more adaptive allele in this temperate region where rice is severely damaged by blast.

Comparison of plants with *Pi35* and *Pish* in terms of their interaction with diverse blast races gives us further insight into the durability of quantitative resistance. As mentioned above, plants with *Pi35* show quantitative and broad-spectrum resistance against pathogen isolates from Japan. But they show a more susceptible phenotype, equivalent to that of plants lacking *Pi35*, against single isolates from China (Yasuda et al. 2008). In contrast, plants with *Pish*, but not *Pi35*, are moderately resistant against all the tested isolates from the Philippines but are highly susceptible to blast in natural field conditions in Japan (Takehisa et al. 2009). From these observations, the mode of action of *R*-genes encoding typical NBS–LRR protein might vary among pathogen populations in different locations, and, therefore, classification of quantitative or qualitative resistance conferred by typical NBS–LRR protein might not be static. Whether the geographical cline of the pathogen will be maintained over time needs to be considered when assessing the durability of resistance conferred by an *R*-gene. Gene flow among geographically separated pathogen populations, sexual reproduction, and high mutation rates can erode quantitative resistance and needs to be considered in the future.

20.3.3 *Enhanced Expression of an* R-*gene,* Pi63*, Confers Quantitative Resistance to Blast*

Identification of the *Pi63* gene uncovered other aspects of quantitative resistance in terms of durability and the magnitude of effect on resistance phenotype. This section focuses on the map-based cloning and characterization of *Pi63* (Xu et al. 2014) and additional information provided by genetic studies of QTLs in the same chromosomal region as *Pi63*.

Pi63 is derived from a Japanese upland rice cultivar "Kahei," which has exhibited a high level of quantitative resistance and durability for nearly a century. Two resistant QTL alleles, *qBFR4-1* and *qBFR4-2*, account for the resistance of "Kahei." *qBFR4-1* is located on chromosome 4 in a region containing an *R*-gene cluster where QTLs for blast resistance have been reported in various cross combinations (Endo et al. 2012; Goto 1988; Hirabayashi et al. 2010; Kato et al. 2002; Miyamoto et al. 2001; Mizobuchi et al. 2014; Terashima et al. 2008; Wang et al. 1994). In addition, a genome-wide association study revealed that SNPs in this chromosomal region are significantly associated with rice blast resistance (Zhao et al. 2011). *qBFR4-2* is located on chromosome 4 around the *Pi21* locus and probably corresponds to *pi21*, because of *Pi21* locus sequence identity between "Kahei" and *pi21*-containing resistant cultivars (Fukuoka et al. 2009). Fine genetic mapping of *qBFR4-1* identified a single locus named *Pikahei-1(t)* within a region of ~300 kb (Xu et al. 2008). Sequencing of three bacterial artificial chromosome clones that cover the *Pikahei-1(t)* locus identified 42 predicted open reading frames (ORFs) including seven RGAs with an NBS–LRR domain; five of these RGAs were expressed in plants carrying the resistant *Pikahei-1*(t) allele. Blast inoculation tests using transgenic rice lines carrying the genomic fragment of each one of five RGAs revealed that only one of the RGAs was associated with blast resistance. Based on these results, *Pikahei-1*(t) was named as *Pi63* and was concluded to encode a typical class of NBS–LRR protein. As found for typical qualitative *R*-resistance genes, an inoculation test using a near-isogenic line (NIL) for *Pi63* and multiple blast isolates identified that *Pi63*-mediated resistance shows race-specificity. This characteristic could not be identified in the genetic background of the donor cultivar owing to the non-race-specific effects of the *pi21* allele and other resistant QTL alleles.

In addition to the difference in amino acid sequences, differences in expression level between the *Pi63* allele in the resistant cultivar and its counterpart allele in a susceptible cultivar are associated with the resistance phenotype, because a marked correlation ($r^2 = 0.63$) was observed between gene expression levels and the level of resistance (evaluated by lesion number per plant) in transgenic lines (Xu et al. 2014). Enhanced *Pi63* expression in transgenic lines was associated with moderate resistance against pathogen isolates that produce the "highly susceptible" phenotype in the NIL for *Pi63*. Therefore, variation in expression levels of genes encoding NBS–LRR proteins could be part of the genetic mechanism responsible for quantitative resistance in rice.

The chromosomal region around the *Pi63* locus includes additional genetic loci for quantitative resistance. Introgression lines carrying a 4-Mb region contain the *Pi63* locus from upland rice cultivar "Owarihatamochi" show quantitative resistance (Fukuoka et al. 2012). This resistance QTL, designated *qBR4-2*, had the second largest effect after *pi21* in an initial QTL mapping study (Fukuoka and Okuno 2001). Fine genetic dissection of this QTL was conducted by using recombinant progeny lines showing recombination within and around *qBR4*-2; these lines were derived from a cross between one of the resistant introgression lines mentioned above and the susceptible cultivar "Aichiasahi" (Fukuoka et al. 2012). The analysis resolved *qBR4-2* into three loci, designated *qBR4-2a*, *qBR4-2b*, and *qBR4-2c*. The locus *qBR4-2b* probably corresponds to *Pi63* based on its physical position, despite the low sequence similarity between "Owarihatamochi (*qBR4-2b*)" and "Kahei (*Pi63*)" in terms of number of ORFs. The locus *qBR4-2a*, which lies 181 kb from *qBR4-2b*, appears to include genes encoding NBS–LRR proteins. Sequence analysis of the donor allele of *qBR4-2a* (i.e., allele derived from "Owarihatamochi") with the corresponding allele in the susceptible "Nipponbare" revealed structural variations in the putative ORFs encoding NBS–LRR domain sequences. The effect of *qBR4-2c* was smallest among the three alleles, but its combination with the donor alleles of *qBR4-2a* and *qBR4-2b* significantly enhanced blast resistance (Fukuoka et al. 2012), as confirmed by marker-assisted gene pyramiding. A study evaluating the effect of *qBR4-2* under multiple environments revealed that *qBR4-2* was less effective at high temperatures than at low temperatures before and/or during infection in field tests (incubation at an average temperature of >25 °C vs. ≤ 25 °C) and in a greenhouse challenge (incubation at 24 °C or 28 °C vs. 20 °C for 1 week before inoculation) (Fukuoka et al. 2015); temperature-sensitive effects of resistance QTLs have also been reported in other pathosystems (Fu et al. 2009; Negeri et al. 2013; Zhu et al. 2010). Change in effect with temperature and/or the expression level of the resistance allele partly explains phenotypic instability of quantitative resistance that breeders should take care to monitor in the use of such QTL alleles.

20.3.4 Atypical NBS–LRR Gene that Shows Age-Dependent Expression and Confers Panicle Blast Resistance

Panicle blast 1 (*Pb1*), a gene derived from the *indica* rice cultivar "Modan," increases resistance to blast during plant development (Fujii et al. 2000), similar to that of wheat cultivars carrying *Lr34* (Krattinger et al. 2009). Plants carrying this gene are blast susceptible during young vegetative stages, but the resistance level increases, as the plants grow, and persists even after heading (Fujii and Hayano-Saito 2007). This gene is a useful source for conferring resistance to panicle blast because cultivars with this gene maintain resistance over several decades (Fujii and Hayano-Saito 2007). Expectations that information about the *Pb1* gene would

uncover unique features of the evolutionary origin and mechanism of age-dependent resistance, at least in part, led to the map-based cloning and characterization of this gene (Hayashi et al. 2010).

Panicle blast resistance is highly sensitive to environmental factors, and thus genetic analysis and selection for this trait are generally difficult. Fortunately, *Pb1*-mediated resistance had been identified from breeding lines carrying *Stvb*^{i}, a gene responsible for resistance to rice stripe virus (Fujii and Hayano-Saito 2007; Shumiya 2002). A genetic study identified the gene responsible for panicle blast resistance (i.e., *Pb1*) on rice chromosome 11 at a distance of 5.8 cM from *Stv-b*^{i} (Fujii et al. 2000). Map-based cloning of *Pb1* showed that it encodes an NBS–LRR protein and is located within one of several tandemly repeated 60-kb units, that is, within an *R*-gene cluster that appears to have been generated as a consequence of recent multiplication and rearrangement (Hayashi et al. 2010). Interestingly, Pb1 belongs to an atypical CC-NBS-LRR class that differs from previously reported R-proteins, particularly in the NBS domain, where the P-loop is apparently absent and some other motifs have degenerated. These motifs are essential for R-protein functions such as nucleotide binding, ATP hydrolysis, and transduction of pathogen perception into R-protein activation (Takken et al. 2006; Tameling et al. 2002; Van Ooijen et al. 2008). So, it is speculated that such characteristics of *Pb1* differentiate its mechanisms for pathogen recognition and downstream signal transduction from those of other R-proteins and could be the key to its durable resistance (Hayashi et al. 2010).

Pb1 transcript levels increase during the development of *Pb1*-resistant cultivars (Hayashi et al. 2010); this expression contributes only partially to resistance to leaf blast but effectively controls resistance to panicle blast. As described above for the *Pi63* gene, overexpression of *Pb1* gene improves resistance to leaf blast, despite the magnitude of effect being limited compared with that of typical *R*-genes. Promoter analysis uncovered that genome duplication event in the region played a crucial role in generating the promoter sequence that produces the *Pb1*-characteristic expression pattern and that this expression pattern, rather than functional differences in the encoded protein, most likely contributes to the resistance (Hayashi et al. 2010). Thus, *Pb1* gene is a case where genomic rearrangement has played a crucial role in generating a new *R*-gene allele in rice, as reported in other plants (Piffanelli et al. 2004; Xiao et al. 2008).

20.3.5 *Analysis of Factors Associated with* Pb1*-Mediated Resistance*

To elucidate the signaling pathway associated with *Pb1*-mediated resistance, the protein–protein interaction between Pb1 and WRKY45, a transcription factor involved in induced resistance via the salicylic acid (SA) signaling pathway, was tested (Inoue et al. 2013). The results suggest that these proteins directly interact

through the CC domain of Pb1. Furthermore, the finding that *Pb1*-mediated panicle or leaf blast resistance is largely compromised when WRKY45 is knocked down in plants carrying the resistant *Pb1* allele suggests that the above interaction is required for the resistance (Inoue et al. 2013). As expected from this working model, overexpressing the *NahG* gene, which encodes a SA-degrading protein, decreases *Pb1*-mediated resistance (Inoue et al. 2013). Rice WRKY45 is regulated by the ubiquitin proteasome system to suppress unnecessary defense reactions in the absence of pathogens and possibly to enhance its transcriptional activity upon activation of the SA pathway (Matsushita et al. 2013). Hence, WRKY45 protein accumulated following addition of a proteasome inhibitor in a protein assay using wheat germ extracts. Interestingly, coexpression of Pb1, instead of addition of the inhibitor, also increased the amount of WRKY45 produced in the assay, possibly by protecting WRKY45 from the ubiquitin–proteasome system (Inoue et al. 2013). Supporting this notion, WRKY45 protein accumulated to a higher level in plants with overexpressed Pb1 than in control plants after a blast challenge (Inoue et al. 2013). These findings suggest that inhibition of WRKY45 degradation is part of the mechanism of *Pb1*-mediated blast resistance.

Despite five typical *R*-gene-encoded proteins (Pi36, Pib, Pita, Pit, and Piz-t; all CC-NB-LRR proteins) having the ability to interact with WRKY45 and WRKY66, resistance conferred by these proteins was not altered by suppression of *WRKY45* or *WRKY66* genes by RNAi (Liu et al. 2016). This result suggests that binding between the CC-NB-LRR protein products of these five genes and WRKY proteins is irrelevant to induction of plant defense. Because WRKY45 is a key factor in the expression of *Pb1*-mediated resistance, as described above, the finding indicates that *Pb1*-mediated resistance belongs to a distinct signaling pathway, despite *Pb1* encoding a CC-NB-LRR protein.

Genetic loci associated with *Pb1*-mediated resistance are targets for clarifying this form of resistance. Breeders have empirically noticed that some cultivars or breeding lines derived from *Pb1*-resistant plants carry a *Pb1*-resistant allele (as judged by DNA markers) but show a more susceptible phenotype. In one such line, "Kanto 209," expression of the *WRKY45* gene is only weakly induced by rice blast inoculation at the full heading stage (Inoue et al. 2017). In "Kanto 209," resistance is enhanced when WRKY45 is activated by application of benzothiadiazole, suggesting that "Kanto 209" has mutations in gene(s) in the signaling pathway upstream of WRKY45 that are required for full induction of *Pb1*-mediated resistance (Inoue et al. 2017; Shimono et al. 2007). To test this hypothesis, QTL mapping was conducted in a population derived from a cross between "Kanto 209" and a cultivar that shows *Pb1*-mediated panicle blast resistance (Inoue et al. 2017). The analysis identified four QTLs on a total of four chromosomes: on chromosomes 7, 9, and 11, susceptible alleles were from "Kanto 209," and on chromosome 8, the resistant allele was from "Kanto 209." These chromosomal regions lack genes encoding enzymes known to regulate WRKY45.

Known genes for such enzymes are rice mitogen-activated protein kinase kinase OsMKK10-2 on chromosome 3 and rice mitogen-activated protein kinases, OsMPK4 and OsMPK6, on chromosome 10, and the protein tyrosine phosphatases OsPTP1/2 (which function in the SA pathway) on chromosome 12 (Ueno et al. 2015). Further analysis is required to fully understand the mechanism of WRKY45 activation associated with *Pb1*-mediated resistance.

20.3.6 Pi34 *Contributes to Blast Resistance by a Mechanism Different to That of Previously Cloned Genes*

Pi34 was identified as a major QTL on chromosome 11 in a genetic mapping population developed from a Japanese breeding line "Chubu 32" (Zenbayashi et al. 2002). Because "Chubu 32" is derived from a *japonica* upland rice cultivar (Koizumi and Fuji 1995), the QTL on chromosome 11 (Kato et al. 2002; Miyamoto et al. 2001), or marker locus significant at the 0.05 probability level in *t*-test (Fukuoka and Okuno 2001) reported by using *japonica* upland and irrigated rice crosses, probably corresponds to *Pi34*; this is confirmed by evidence concerning the identity of the DNA haplotypes in or near the region containing *Pi34* in the "Chubu 32" and *japonica* upland rice cultivars "Sensho" and "Owarihatamochi" used in those studies (Fukuoka, unpublished data). Although the contribution of *Pi34* to reduction in lesion area is smaller than that of *Pi63* or *pi21*, less background noise in the mapping populations used has allowed researchers to develop a NIL for *Pi34* and to clarify the phenotypic characteristics of *Pi34*-mediated resistance. Interestingly, this gene restricts development of lesions, so after challenge by compatible fungus races, plants carrying the resistant *Pi34* allele have less lesions than observed in plants lacking this allele (Fukuoka et al. 2015; Zenbayashi-Sawata et al. 2007). The penetration frequency of fungus into the epidermal cells (>90%) did not differ among plants carrying *pi21*, *Pi35*, or *Pi34* at 40 h after inoculation; the number of leaf sheath cells at 40 h after inoculation was lowest in plants carrying *Pi34*; and, at 6 d after spray inoculation, the number of sporulating lesions (an indicator of failure to prevent hyphal growth) was largest in those carrying *Pi34* (Yasuda et al. 2015). This data suggests that *Pi34* prevents hyphal invasion but not the development of lesions at later stages of infection.

Pi34-mediated resistance shows race-specificity, with plants carrying *Pi34* demonstrating less resistance to the race "IBOS8-1-1" than to other races (Koizumi and Fuji 1995). Genetic analysis of a segregating population resulting from a cross between blast races "IBOS8-1-1" and "Y93-245c-2" indicated that "Y93-245c-2" carries an avirulence gene to *Pi34*, which was called *AVRPi34*, and that "IBOS8-1-1" is extremely aggressive on plants carrying *Pi34* because of the absence of

AVRPi34 (Zenbayashi-Sawata et al. 2005); this study was the first report of a gene-for-gene relationship between a fungal disease resistance gene associated with severity of disease and pathogen aggressiveness. Fine genetic mapping of *Pi34* delimited this locus to a region of less than 100 kb containing ORFs that lack similarity with previously characterized blast resistance genes (Zenbayashi-Sawata et al. 2007). Further genetic mapping and screening of the genes in the *Pi34* locus that are differentially expressed between a susceptible cultivar and its NIL for *Pi34* identified a previously uncharacterized gene whose reduction in expression by RNAi increased susceptibility to blast (Kito and Zenbayashi-Sawata 2012). Identification of *AVRPi34* and map-based cloning and functional characterization of *Pi34* are expected to provide new insights into rice–blast interactions.

20.4 Marker-Assisted Gene Pyramiding for Durable Resistance

As discussed above, durably resistant rice genotypes carry multiple resistant alleles. MAS is a method by which breeders can efficiently transfer one or more of these resistant alleles into elite genetic backgrounds. Despite the many reports of QTL alleles for quantitative resistance to blast, some bottlenecks hinder the development of durably resistant cultivars by MAS. This section explains two key issues, linkage drag and assessment of combined effect, that need to be considered in marker-assisted gene pyramiding for durable resistance.

20.4.1 Removal of Linkage Drag

DNA markers can be effectively used in breeding to eliminate linkage drag, which is the co-introduction of beneficial and undesirable agricultural traits from exotic genetic resources (Fukuoka et al. 2009). Since the donors for quantitative resistance usually have unimproved genotypes, their morphological and physiological characteristics are usually distinct from cultivars with elite genotypes; therefore, dealing with linkage drag is important for efficient improvement of quantitative resistance in breeding programs. Cultivars of *japonica* upland rice have been extensively used in breeding since the 1920s, but the undesirable characters of the donors limit the introduction of genes for quantitative resistance into such elite cultivars (Morimoto 1980). Developing near-isogenic lines (NILs) for each quantitative resistance QTL in a desirable genetic background is one strategy for overcoming this problem.

During MAS, the size of the introgression around the target QTL needs to be minimized to reduce the risk of linkage drag. However, when the precise map position of the target has not been determined, the size of introgression will be unnecessarily large so as not to miss the gene. The durable blast resistance gene *pi21* was found to be linked with gene(s) associated with inferior eating quality within a 40-kb distance (Fukuoka et al. 2009). In that study, desirable recombinants between *pi21* and the genes conferring inferior eating quality were successfully selected from a large breeding population by using DNA markers for the genes around *Pi21* (Fig. 20.3). The availability of DNA markers closely linked to the genes of interest enabled a breakthrough in the development of durably resistant cultivars that had not been achieved during 80 years of conventional breeding in Japan. Thus, this is a clear example of selection in breeding that could not have been achieved without the use of DNA markers tightly linked to the genes and DNA regions of interest. The recessive nature of *pi21* would have also made this allele difficult to introduce into elite cultivars by conventional selection procedures.

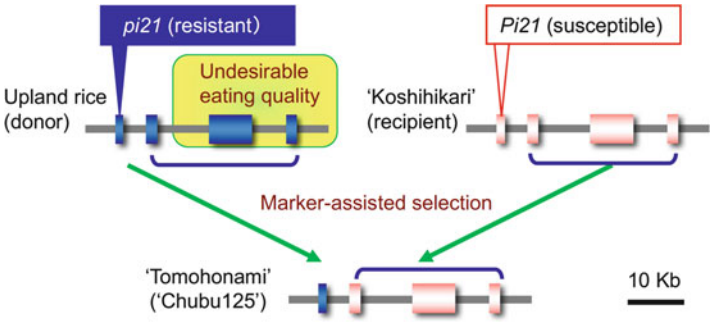

Fig. 20.3 Removal of undesirable traits that are tightly linked with the *Pi21* locus whose recessive allele confers resistance to blast. *Pi21* and three genes downstream from *Pi21* are indicated by boxes. Based on the locations of the genes for *Pi21* and eating quality, individual plants with a recombination event downstream of the *Pi21* locus were selected, and progenies that carried homozygous alleles for resistance (i.e., *pi21* from an upland rice cultivar "Sensho") and desirable eating quality (from an elite cultivar "Koshihikari") were selected from a breeding population by marker-assisted selection (MAS). Since these genes are located within a 40-kb region, desirable individuals were obtained at a rate of 1 in 2000. Note that quantitative resistance and eating quality are environmentally unstable traits that require progeny testing for reliable measurement. Additionally, because of the recessive nature of the *pi21* allele, plants that are heterozygous at the *Pi21* locus show the susceptible phenotype, like that of plants with homozygous *Pi21* alleles. Therefore, it is difficult to select desirable individuals in the field. So, MAS has provided a breakthrough in the development of resistant cultivars that has not been achieved by 80 years of conventional rice breeding

20.4.2 Assessment of the Combined Effect of a Gene Pyramid

DNA markers are a powerful tool for combining multiple alleles for blast resistance, because it is difficult to judge the number of resistance alleles by phenotype alone. Combining multiple QTL alleles is expected to enhance the blast resistance, since most resistance QTL alleles additively contribute to reduction of lesion area. Breeders and researchers know that disease resistance partly interacts with genetic backgrounds and/or environmental factors (Hashioka 1985; Matsuyama and Dimond 1973; Ohata et al. 1966; Sun et al. 2004; Yunoki et al. 1970). Evidence provided in this review supports this idea in the contexts of race-specificity and temperature-dependent resistance and the presence of genetic loci that modulate induction of resistance or its mode of action (Fukuoka et al. 2015; Inoue et al. 2017; Zenbayashi-Sawata et al. 2005). Therefore, to understand how respective resistant alleles interact with such factors, it is important to determine the appropriate number and combinations of resistance genes. However, knowledge of the impact of QTL pyramiding on the robustness of plant defense in rice has been limited.

This section describes a study that demonstrates the potential of QTL pyramiding by combining six resistance alleles at four loci, *pi21*, *Pi34*, *qBR4-2* (*qBR4-2a*, *qBR4-2b*, *qBR4-2c*), and *qBR12-1*, to produce a durably resistant genotype with a strong, non-race-specific defense response (Fukuoka et al. 2015). This study highlights the importance of minor QTL alleles for improving the durability of resistance by using experimental lines with a highly homogeneous genetic background. The average reduction of lesion area of NILs, respectively, carrying *pi21*, *Pi34*, *qBR4-2*, or *qBR12-1* compared with recurrent parents in eight field trials was 87%, 39%, 45%, and 22%, respectively. As expected, the resistance conferred by each of the four alleles individually was substantially sensitive to environmental factors, and the effect of minor QTL alleles was sometimes undetectable. However, combinations of these resistance alleles dramatically reduced lesion area both in field tests and in greenhouse inoculation tests. The line with four resistance alleles had lesion areas of 1% or lower, which is comparable to the level in the donor and only 6% of the level in the line carrying the major allele *pi21* alone. Importantly, the coefficient of variation for lesion area across field tests was smaller in the line with four resistance alleles than in lines with only one or two resistance alleles. In contrast, the resistance of the NILs that each carry a single qualitative *R*-gene—*Pik*, *Pik-m*, *Piz*, *Piz-t*, *Pita*, *Pita-2*, *Pib*, or *Pii* —fluctuated in field tests, possibly owing to changes in pathogen populations over time. These observations strongly suggest that QTL pyramids confer more robust resistance than that provided by *R*-genes or by any single QTL.

Histological examination revealed that the four-QTL pyramid line has no hypersensitive response that is usually associated with qualitative resistance (Fukuoka et al. 2015). Furthermore, induction of pathogenesis-related genes was not as great

as that induced by qualitative resistance at the tested time points. Increased expression of pathogenesis-related genes at around 32–36 h after inoculation, and lower cell viability (even without signs of cell death) at 33 h after inoculation, in the four-QTL pyramid plants compared with the same time point in no-QTL or *pi21*-only plants suggests induction of the defense response earlier in these plants than in no-QTL or *pi21*-only plants. These observations suggest that enhanced resistance in the four-QTL pyramid plants does not mimic qualitative resistance but does allow plants to effectively suppress fungal development in the cell.

Resistance of the cultivar carrying four genes for qualitative resistance was rapidly overcome by compatible pathogen races (Kiyosawa 1989). Additional evidence regarding this point will be provided by monitoring of the resistance of the line carrying *Piz-t* and *Pi54* developed by Xiao et al. (2017). Yet, drastic change of pathogen population structure is widely understood to occur when cultivars are replaced by those with different *R*-genes, as suggested by an epidemiological survey and simulation study on pathogen race dynamics (Ashizawa et al. 2015). Such observations imply that a pyramid of *R*-genes, each posing strong selection pressure against the pathogen, may not improve the durability of resistance. Thus, the findings on QTL pyramids are important for plant breeders because trait stability is a key requirement for sustainable production of agricultural crops.

20.5 Conclusion and Future Work

As well as their involvement in diverse biological functions, genes for quantitative (QTL-mediated) resistance contribute to more durable resistance than those involved in qualitative (*R*-gene mediated) resistance. As previously discussed, some genes for quantitative resistance could be weak alleles of *R*-genes, and some display race-specificity, temperature-dependence, and/or age-dependence. The incomplete resistance conferred by quantitative resistance genes might decrease the selection pressure on pathogens and slow their counter-evolution. It is desirable to use multiple resistant alleles in combinations to ensure a more environmentally stable and stronger resistance compared with that of a single resistance allele. Precise map information of resistant alleles should be obtained to avoid the issue of linkage drag in practical breeding programs. The workflow used to develop quantitative resistance in elite cultivars is depicted in Fig. 20.4. The biological cost of resistance should be evaluated in the future. Unlike the case of barley mutant *Mlo* (Brown 2002; Buschges et al. 1997), NILs carrying single quantitative resistance alleles do not display a yield penalty (Fukuoka et al. 2009); however, the yield penalty of rice plants with enhanced defense through multiple resistant alleles has not been well clarified. Although plants with multiple

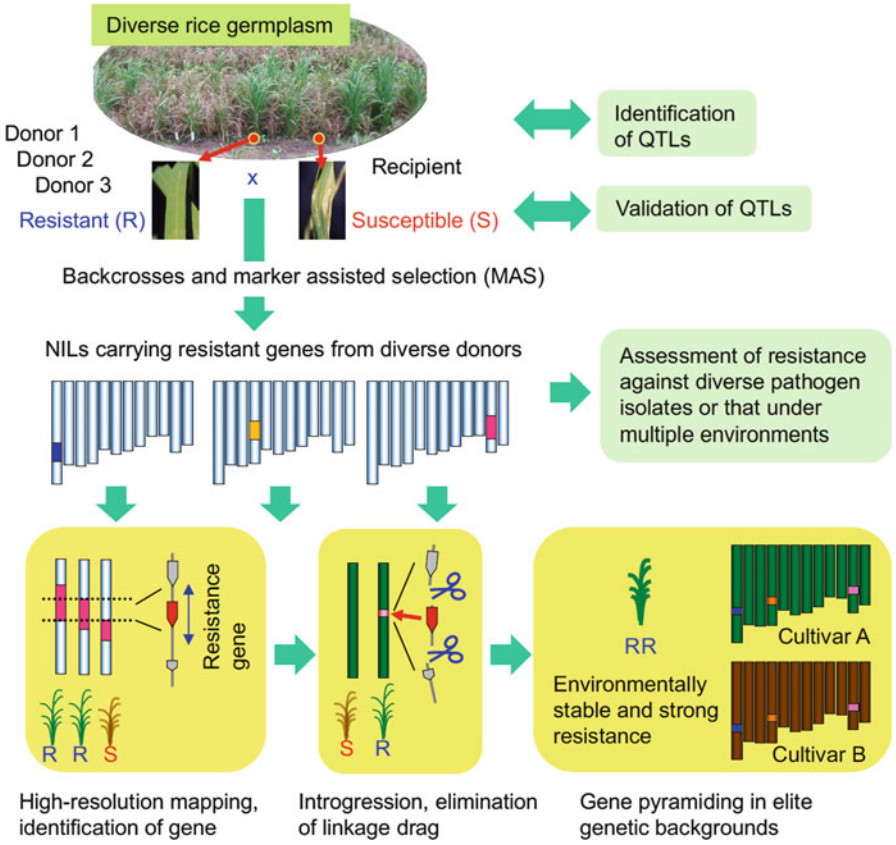

Fig. 20.4 A marker-assisted backcross breeding strategy for quantitative resistance to blast. Cultivars or landraces that show quantitative resistance to blast are backcrossed with a susceptible genotype (ideally an elite cultivar), followed by QTL analysis and validation of the QTL in the progeny lines. After two or three rounds of these processes, near-isogenic lines (NILs) each containing a single QTL from one of the donors are produced and used to characterize the respective QTLs in terms of resistance spectrum and/or environmental stability. NILs are further used for high-resolution mapping and identification of the relevant genes. Map information for the respective genes is useful for their introduction into the elite genetic background without risk of linkage drag. Finally, breeding lines with improved blast resistance are crossed with each other to develop QTL pyramids that show environmentally stable and strong resistance to blast

resistant alleles do not show deleterious penalties on plant growth under standard cultivation conditions (Fukuoka, unpublished data), further evaluation under multiple environments is required to answer this question.

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Chapter 21
Genes Affecting Eating and Processing Qualities

Takayuki Umemoto

Abstract The eating and processing qualities of rice are major targets in rice breeding. Rice quality is evaluated multidimensionally, including the appearance, texture, taste, and aroma of cooked rice. Among these qualities, texture is of the greatest interest in rice-consuming countries. The textural attributes of cooked rice have traditionally been evaluated in terms of the amylose content (AC), gel consistency (GC), and alkali spreading score (ASS) of the grain. Of the enzymes involved in the biosynthesis of rice starch, granule-bound starch synthase I (GBSSI, Wx), starch synthase IIa (SSIIa, Alk), and starch-branching enzyme I (BEI) receive the most attention, since natural variations and induced mutations in the genes coding for these enzymes are tightly linked to the textures of cooked and processed rice. The present review focuses on starch, the largest component of rice, and the predominant determinant of the texture of cooked and processed rice. In particular, recent advances in research on the regulation of these important quality parameters are surveyed, and their applications to breeding programs illustrated.

Keywords Amylose · Amylopectin · Gelatinization · GBSS · SNP · Starch · Starch-branching enzyme · Starch synthase · Wx

21.1 Introduction

Rice quality is evaluated primarily on the appearance, texture, and aroma of the cooked grain (Fitzgerald et al. 2009). Among these, the cooked rice texture, which is largely determined by the starch properties of the grain, is of the greatest interest in eating quality evaluations in rice-consuming countries. The textures of rice-based processed foods, such as noodles, rice cakes, and rice flour bread, are also affected by the properties of rice starch.

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Three types of parameters have traditionally been used to estimate rice cooking and processing qualities: amylose content (AC), gel consistency (GC), and alkali spreading score (ASS), all of which are strongly related to the starch properties. The AC is the ratio of amylose to starch and is often expressed, in breeding programs, as the ratio of amylose to flour. The AC generally correlates positively with the hardness, and negatively with the stickiness, of cooked rice (Juliano et al. 1981; Juliano and Villareal 1993). The GC also indicates the hardness of cooked rice, especially among the high-amylose class of rice (Cagampang et al. 1973). The ASS is negatively correlated with starch gelatinization temperature and hence with the time and energy needed for rice to be cooked (Juliano 1985).

This review begins with a brief characterization of rice starch and then turns to recent advances in the genetic and molecular understandings of the abovementioned three parameters, together with their application to breeding programs. In addition, the QTL analyses of eating quality of Japanese leading cultivars are also summarized. Extensive studies on each class of starch biosynthetic enzyme and the physical association necessary for proper starch formation, as well as the emerging interest in rice with high-resistant starch for the health benefits, have been reviewed in other reports (Fujita 2014; Tetlow 2011; Rahman et al. 2007; Tsuiki et al. 2016; Zhou et al. 2016).

21.2 Rice Starch Composition and Structure

Cereal starches, including rice starch, are composed of amylose and amylopectin, both of which are α-glucans with highly polymerized glucose residues, but have distinct molecular structures. Amylose is mainly a linear α-glucan, with glucose residues combined by α-1,4 linkages, and carrying a few branch chains generated by α-1,6 linkages (Takeda et al. 1986). The number-average degree of polymerization (DPn) of 860–1,240 for rice amylose is comparable to other cereal starches such as wheat, barley, and maize and less than that of root and tuber starches (e.g., sweet potato, with a DPn of roughly 4,000) (Hanashiro 2015). A granule-bound starch synthase I (GBSSI, Waxy, or Wx protein) is indispensable for producing amylose, because the absence of the Wx protein results in amylose-free glutinous (waxy) rice (Sano 1984). Amylopectin has a cluster structure with highly and regularly branched chains, which are formed by α-1,6 linkages on the α-1,4-linked linear chains. The molecular size of amylopectin is one order larger in DPn than amylose (Takeda et al. 2003). Three classes of enzymes, starch synthase (SS), starch-branching enzyme (BE), and starch-debranching enzyme (DBE), work in concert for the biosynthesis of amylopectin (Nakamura 2015). SS elongates the linear chains of α-1,4 linkages, BE cuts linear chain segments and attaches them to the side of linear chains by α-1,6 linkages, and DBE debranches the improperly attached α-1,6-linked side chains to maintain the highly organized amylopectin structure, with semicrystalline and amorphous layers of the starch granules. The amylopectin chains of DP (degree of polymerization) $\geq$10 form

double helixes and packed together to generate semicrystalline layers (Tetlow 2011). This enables cereal seeds to conserve a condensed energy source for the next generation in a limited space, and humans reap great benefits from this energy, in the form of staple foods.

21.3 The *Wx* Gene: Its Roles in Amylose and Amylopectin Synthesis

21.3.1 Major Alleles: Wx^a, Wx^b, and wx

The apparent AC of rice cultivated worldwide shows wide variation. Rices are classified as waxy (0–5%), very low (5–12%), low (12–20%), intermediate (20–25%), or high (25–33%) based on the amylose percentage of starch as indicated (Juliano and Villareal 1993). Asian regional preferences for the amylose type have been reported (Calingacion et al. 2014). In spite of the wide variation of amylose contents, ssp. *indica* cultivars tend to have higher, and ssp. *japonica* cultivars tend to have lower, amylose content (Nakamura et al. 2002).

The above difference in amylose content between the two subspecies of rice is mainly determined by the *Wx* alleles, Wx^a and Wx^b (Sano 1984). The *Wx* gene codes GBSSI, the enzyme essential for amylose synthesis in rice endosperm. *Indica* rice generally has Wx^a allele, with the capability of producing about ten times more Wx protein (GBSSI) than the Wx^b of *japonica* rice. The functional differences in the Wx^a and Wx^b alleles are due to the fact that a "G" or "T" SNP (single-nucleotide polymorphism) lies in the 5′ splice site of the first intron. The SNP has changed the splice site from AG**G**T of Wx^a to AG**T**T of Wx^b, which leads to aberrant pre-mRNA splicing, resulting in less mature *Wx* mRNA and reduced amounts of Wx protein and, consequently, reduced amylose content (Wang et al. 1995; Cai et al. 1998; Isshiki et al. 1998). Wx^a is a wild-type allele distributed widely among *Oryza rufipogon* and *Oryza nivara*, wild relatives of cultivated rice, and another cultivated rice species, *Oryza glaberrima*, in addition to *Oryza sativa* ssp. *indica* (Sano et al. 1986; Yamanaka et al. 2004). Based on the evolutionary relationship, Wx^b is thought to have originated from *Oryza rufipogon* and to have spread to the population during the domestication of *Oryza sativa* ssp. *japonica* (Hirano et al. 1998).

Another major *Wx* allele is *wx*, a null allele. The lack of Wx protein caused by the mutation in the *Wx* gene results in amylose-free, waxy, or glutinous rice. The texture of cooked glutinous rice is very sticky compared to that of non-waxy (non-glutinous) rice. Waxy rice is traditionally used to make rice cakes, steamed rice, and sweets including ceremonial foods. However, waxy rice is also consumed as a staple food in East Asian countries, including Laos and northern regions of Thailand known as the "center of the glutinous rice zone" (Watabe 1967; Calingacion et al. 2014; Juliano and Villareal 1993). Natural variations involving 23-base duplication in the second exon of the *Wx* gene have been found in glutinous

rice cultivars (Inukai et al. 2000; Wanchana et al. 2003). This duplication generates a premature early stop codon and results in the absence of functional Wx protein. This variation probably occurred in the *japonica* group, because all the glutinous rice possessing 23-base duplication also has the "T" SNP in the first intron of the *Wx* gene, the signature of Wx^b. However, 29 of the 35 waxy rice cultivars classified as *indica* also possess the Wx^b-derived null allele (Yamanaka et al. 2004), which indicates introgression of the *japonica* null *wx* allele to *indica* (Muto et al. 2016). The null *wx* allele with 23-base duplication has spread outside the glutinous rice zone, for example, in China, Bangladeshi, Taiwan, Japan, Thailand, and the United States (Chen et al. 2014; Shahid et al. 2016; Inukai et al. 2000; Mikami et al. 2008; Wanchana et al. 2003; Hori et al. 2007; Biselli et al. 2014).

21.3.2 Amylose Content Is Affected By the Temperature During Grain Filling

High temperatures during grain filling result in a decrease of rice grain AC (Asaoka et al. 1984). AC of the Wx^b cultivars and Wx^b-based low-amylose cultivars is more susceptible to the changes in temperature during grain filling (Fig. 21.1). Both the amount of Wx protein and GBSSI activity decrease with an increase in temperature (Hirano and Sano 1998; Umemoto and Terashima 2002; Inukai and Hirayama 2010). The temperature sensitivity of AC in the Wx^b cultivars during grain filling is due to decreased steady-state levels of the *Wx* mRNA. The processing efficiency of the *Wx* pre-mRNA is lower at higher temperature, and this lower processing efficiency is caused by the "G" to "T" SNP at the splice donor site of the first intron of *Wx* gene (Hirano and Sano 1998; Larkin and Park 1999).

21.3.3 GBSSI Synthesizes Long Chains of Amylopectin

For a long time after the role of the Wx protein (GBSSI) in amylose synthesis in rice was established (Sano 1984), its role in extra-long- or super-long-chain (LC) synthesis of amylopectin was overlooked. The presence of LCs in amylopectin, with chain length ≥ 100, was reported by Takeda et al. (1987). Three *indica* cultivars with apparent amylose content of 30–32% actually contained only 15.5–18.5% amylose, with the rest accounted for by the contribution of very long chains of amylopectin. The proportion of amylopectin LCs is clearly correlated to the amount of Wx protein bound to starch granules (Inouchi et al. 2005) (Fig. 21.1). A role of GBSSI in amylopectin LC synthesis was tested by screening and characterizing a *wx* null mutant from the mutanized population of a cultivar possessing Wx^a with high LC content (Aoki et al. 2006). The *wx* mutant completely lacked LCs, while the original cultivar had 11.3% LCs in its amylopectin. Hanashiro et al. (2008)

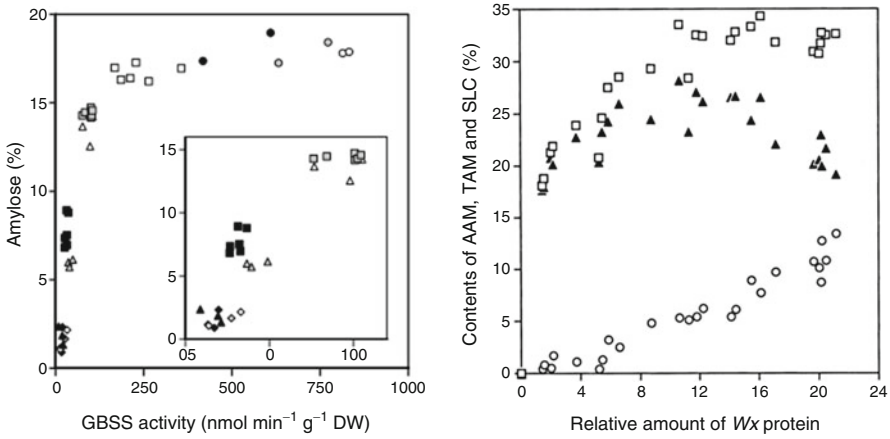

Fig. 21.1 Relationship between GBSS activity, amount of Wx protein, and amylose content in rice grain. Left: GBSS activity in the endosperm developing under different temperatures and amylose ratio measured as the first fraction of debranched starch by an isoamylase separated using a Sephadex G-75 column. Two Wx^a cultivars (circles), six Wx^b cultivars (squares), three Wx^b-based low-amylose cultivars (triangles), and two waxy cultivars (diamonds). Samples matured under low (18 °C day/13 °C night, open symbols), medium (25/20 °C, shaded), and high (35/30 °C, closed) temperatures. Inset is an enlargement of a part of the figure. Reproduced from Umemoto and Terashima (2002), with permission from CSIRO Publishing. Right: relative amount of Wx protein in starch granules and ratios of apparent amylose (AAM, open square), true amylose (TAM, closed triangle), and super-long chain of amylopectin (SLC or LC, open circle) Reproduced from Inouchi et al. (2005), with permission from Japanese Society of Applied Glycoscience

confirmed the role of GBSSI in LC synthesis by introducing Wx^a transgene into waxy cultivars. The transgenic lines produced 21.6–22.2% apparent amylose (true amylose + LCs), with a significant number of LCs in the amylopectin (7.5–8.4% of amylopectin weight). Together, these studies clearly demonstrated the essential roles of GBSSI in amylopectin LC synthesis.

21.3.4 Wx Gene SNP Is Associated with Gel Consistency and Viscosity Through Changes in Amylopectin LC Level

A gel consistency (GC) test is a simple method developed to estimate the firmness of cooked rice, especially among high-amylose rice cultivars (Cagampang et al. 1973). A number of QTL analyses for GC have been performed using mapping populations derived from parents with differing amylose class combinations, and in many cases, a major QTL was detected around the *Wx* locus (Bao et al. 2002; He et al. 2006; Wang et al. 2007; Lanceras et al. 2000; Tian et al. 2005; Zheng et al.

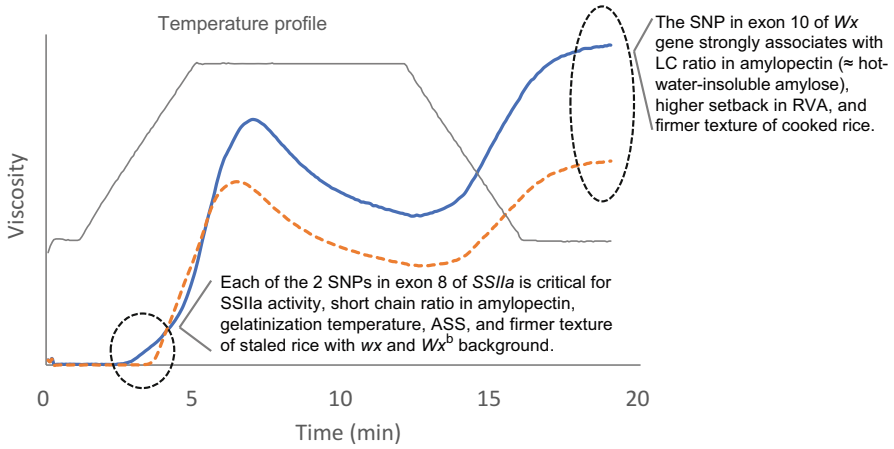

Fig. 21.2 Schematic presentation of RVA profiles of rice flour from the two cultivars Yumetoiro (solid line) and Koshinokaori (dotted line) with contrasting *Wx* and *SSIIa* haplotypes (Data courtesy of Dr. N. Aoki). Both Yumetoiro and Koshinokaori are Wx^a cultivars with apparent AC of ~30%. Yumetoiro has the "T" SNP in exon 10 of *Wx* and "G-T" SNPs in exon 8 of *SSIIa*, while Koshinokaori has "C" SNPs and "G-C" SNPs for the corresponding position of the genes

2008). Su et al. (2011) performed map-based cloning and a complementation test for a gene responsible for the GC differences in a *japonica* (Wx^b) and *indica* (Wx^a) cross and confirmed that *Wx* is the responsible gene. Further, Tran et al. (2011) investigated an F_6 population derived from a cv. IR8 (low GC, hard gel)/cv. IR5 (high GC, soft gel) cross, both of which cultivars have Wx^a and high AC, and revealed that a SNP in exon 10 of the *Wx* gene, "T" or "C," was highly associated with the GC differences. They further confirmed this association using a diverse set of traditional cultivars with an AC of over 25%. In addition, lower GC cultivars tend to have more hot-water-insoluble amylose than higher GC cultivars, and this hot-water-insoluble amylose is thought to broadly correspond to amylopectin LC levels (Reddy et al. 1993).

On the other hand, the SNP in *Wx* exon 10, observed especially within high-amylose cultivars, well explains differences in RVA (Rapid Visco Analyzer) cool paste and setback viscosities, which are indicators of increased retrogradation of gelatinized starch and hence of cooked rice firmness (Chen et al. 2008a) (Fig. 21.2). When the SNP genotype was "T" instead of "C," the viscosities of both cool paste and setback were increased by ~150 and ~60 rapid visco units (RVU), respectively (Chen et al. 2008a). Also, large differences in setback viscosities among high-amylose cultivars reflect differences in the proportion of amylopectin LCs (Horibata et al. 2004). To summarize, it has been shown that amylopectin LC level is the major factor affecting cooked rice textures other than AC as mentioned in the earlier studies (Bhattacharya et al. 1978; Sowbhagya et al. 1987; Reddy et al. 1993). The amino acid change from Ser to Pro caused by the SNP in *Wx* exon 10 is in the glycosyltransferase domain of GBSSI. This mutation would be predicted to alter GBSSI glucan elongation or binding to the starch granule (Tran et al. 2011). It

is also possible that another genetic factor, the occurrence of linkage disequilibrium with the SNP, may affect the expression level of the *Wx* gene and thereby increase the biosynthesis of amylopectin LCs instead of true amylose.

21.4 *SSIIa* Variations Affect Amylopectin Structure and Rice Quality

21.4.1 Natural Variations in SSIIa Gene and Alkali Disintegration of Rice Grain

Differences in amylopectin chain-length distribution among rice cultivars were characterized by size-exclusion chromatography of isoamylase-debranched gelatinized starch (Hizukuri et al. 1989). Three *japonica* cultivars had starches with a higher proportion of short chains (average DP of 16–17) compared to the five *indica* cultivars studied. Later, clear differences in the chain-length distribution of amylopectin between *indica* and *japonica* cultivars were reported, based on high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Umemoto et al. 1999). *Japonica* cv. Kinmaze had a higher short-chain (DP 5–12) to middle-length-chain (DP 13–24) ratio than *indica* cv. IR36. This difference in chain-length distribution was genetically analyzed with a backcross inbred population derived from a cross of *japonica* cv. Nipponbare and *indica* cv. Kasalath, and a major gene *acl* (amylopectin chain length) was mapped to the short arm of chromosome 6 at the same locus as *alk* (Umemoto et al. 2002). The *alk* gene has a large effect on alkali disintegration of rice grains (Kudo 1968; Harushima et al. 1998). In general, polished grains of *japonica* cultivars are susceptible to an alkaline solution of 1.7% (w/v) potassium hydroxide, in which the grains become swollen or disintegrated, whereas many of *indica* cultivars show greater tolerance and grain shape does not collapse (Little et al. 1958). The degree of alkali disintegration (alkali spreading score; or ASS) negatively correlated well with the gelatinization temperature (Juliano et al. 1982) (Fig. 21.2). In the first related report in 1914, it was already suggested that alkali disintegration of rice grains correlated with the starch property (Warth and Darabsett 1914). The relation between ASS and starch structure (i.e., chain-length distribution of amylopectin) has been demonstrated, and one of the starch synthase genes, *SSIIa*, has been mapped to the *acl*/*alk* locus (Umemoto et al. 2002). The identity of *SSIIa* as *alk* has been verified by map-based cloning of the *alk* gene (Gao et al. 2003), and two critical natural variations resulting in nonfunctional *SSIIa* alleles with *japonica* cultivars have been reported (Nakamura et al. 2005; Umemoto and Aoki 2005; Waters et al. 2006; Bao et al. 2006). Both SNPs are positioned in exon 8 of *SSIIa*, G/A at 4196, and C/T at 4328 from the translation start site, causing an amino acid change from Val-737 to Met and Leu-781 to Phe, respectively. Each of the SNP genotype, 4196G and 4328C, respectively, is functional and negatively correlated

to the ASS of rice grain. Both amino acid residues are in the catalytic domain of the enzyme and are conserved as Val and Leu, respectively, among maize, wheat, and barley (Umemoto and Aoki 2005). Does the *japonica* SSIIa completely lack its function? The question is still open for discussion, because both *japonica* alleles produce the SSIIa protein, although they lack the activity and ability to bind tightly to the starch granule (Umemoto et al. 2004; Nakamura et al. 2005). This *japonica*-type SSIIa might retain the ability to form protein complex with starch synthase I (SSI) and starch-branching enzyme IIb (BEIIb) (Luo et al. 2015) and might have some contribution to the amylopectin synthesis, which should be further studied.

21.4.2 Variations in SSIIa Affect Retrogradation of Cooked and Processed Rice

Studies on the effects of functional differences in SSIIa on rice processing and eating qualities have been conducted (Okamoto et al. 2002; Umemoto et al. 2008). The effects of allelic differences of *SSIIa* on the hardening of rice cakes were evaluated using an F_4 population derived from a cross of glutinous cultivars with functional and nonfunctional SSIIa (Okamoto et al. 2002). Rice cakes were made from individual rice plants homozygous for functional and nonfunctional SSIIa and the heterozygotes. After being cooled overnight, rice cakes made from rice with functional SSIIa were clearly harder than those made from nonfunctional SSIIa; and those from heterozygous plants showed moderate hardness, intermediate between that of the former two. A negative correlation between ASS and the hardness of cooked glutinous rice measured by an Instron food tester was investigated in an earlier study (Villareal et al. 1993). Two glutinous *indica* cultivars with ASS 3.0 (high-gelatinization-temperature type) were three times harder than 12 other cultivars, including *indica* and *japonica* with ASS 6.0–7.0 (low-gelatinization-temperature type), in a comparison of staled cooked rice. It is highly probable that the low-ASS cultivars examined by Villareal et al. (1993) had the functional SSIIa, while those with high ASS had nonfunctional SSIIa.

The effects of functional differences in SSIIa (Alk), together with those of GBSSI (Wx) and starch synthase I (SSI), on the eating quality of non-glutinous rice were investigated utilizing near isogenic lines (NILs) (Umemoto et al. 2008) (Fig. 21.3). The NILs had a chromosomal segment that included one of the starch synthase gene loci of *indica* cv. Kasalath (Wx^a/*Alk*) with a *japonica* cv. Nipponbare (Wx^b/*alk*) genetic background. Sensory evaluation of the cooked rice by Japanese testers was performed twice: when freshly cooked and after cooling at 5 °C overnight. The freshly cooked rice of NIL(Wx^a) was clearly less sticky, less glossy, of harder texture, and significantly inferior in overall preference to Nipponbare. This pattern was repeated when the cooked rice was cooled and staled. The variations in SSI between the two cultivars had minor effects on eating quality.

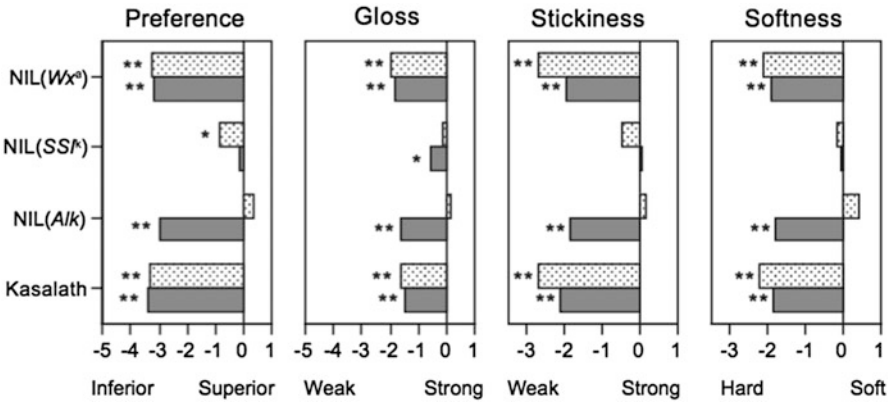

Fig. 21.3 Effects of allelic variations in the three starch synthase genes on the eating quality of cooked rice were evaluated using NILs. The NIL(Wx^a), NIL(SSI^k), and NIL(*Alk*) have a chromosomal segment from an *indica* cv. Kasalath including *GBSSI*, *SSI*, and *SSIIa* locus, respectively, introgressed into a *japonica* cv. Nipponbare genetic background. The cooked rice of the recurrent parent Nipponbare was used as the standard. Overall preference and gloss were scored from −5 to +5 and stickiness and softness from −3 to +3. Samples were evaluated by 19 trained testers 0.5 h after cooking (dotted bars) and after 16 h storage at 5 °C (gray bars). $*P = 0.05$, $**P = 0.01$ relative to Nipponbare by the sign test. Reprinted from Umemoto et al. (2008), https://doi.org/10.1626/pps.11.472

Most interestingly, functional differences of SSIIa scarcely affected the eating quality when the rice was freshly cooked. However, the evaluation of NIL(*Alk*) was dramatically different after staled overnight, with all the items (gloss, stickiness, softness, and overall preference) being significantly decreased compared to those of Nipponbare (Umemoto et al. 2008). Gidley and Bulpin (1987) reported that shorter α-glucan chains (DP < 10) were difficult to recrystallize upon retrogradation. This could explain the quality of NIL(*Alk*) cooked rice, which has a lower proportion of short chains with DP 5–11, and more chains with DP 12–23, compared to Nipponbare. However, these differences in cooked rice properties, based on the SSIIa function, can only be seen clearly in rice with a low-amylose (Wx^b) or glutinous (*wx*) background, but not in those with a high-amylose (Wx^a) background. The cooked rice texture of cv. Kasalath, the donor of Wx^a and *Alk* for the NILs, had a firm texture, both freshly cooked and after staling; and the changes in texture during cooling, due to the function of SSIIa (*Alk*), were masked by the high-amylose properties caused by the Wx^a allele (Fig. 21.3).

The allele-specific DNA markers for *SSIIa* are available (Bao et al. 2006; Cuevas et al. 2010; Nakata et al. 2017). Rice breeders and traders can select preferable texture types for the consumers and food companies by applying DNA marker selection for *SSIIa* alleles, together with the *Wx* alleles (Bligh et al. 1995; Yamanaka et al. 2004; Wanchana et al. 2003; Chen et al. 2008b; Tran et al. 2011).

21.4.3 BEI Deficiency: A Possible Resource to Adjust the Texture of Rice-Based Foods

Three isoforms of starch-branching enzymes, BEI, BEIIa, and BEIIb, are active in the developing rice endosperm (Yamanouchi and Nakamura 1992; Nakamura 2002). Among them, BEI appears to produce longer chains of amylopectin than BEIIa and BEIIb. An in vitro assay of purified rice BE isoforms expressed in *E. coli* suggested that the BEI (RBE1) transferred longer chains, most preferentially DP 10–11, compared to BEIIb (RBE3) and BEIIa (RBE4) (DP 6–7), when potato amylose was used as the substrate (Mizuno et al. 2001). The null BEI mutants, in particular, had a higher ratio of short chains (DP $<$ 12) and a lower ratio of middle-length chains (16 $<$ DP $<$ 23) (Satoh et al. 2003). This probably reflected the differences of BEI vs. BEIIb and BEIIa activity. The gelatinization temperatures of the BEI mutants, as measured by differential scanning calorimetry (DSC), were roughly 5 °C lower than the original cv. Kinmaze. Some agronomically important grain characteristics (grain weight, size, shape, and chalkiness) seem not to be affected by a lack of BEI. In this regard, BEI mutations could be a suitable resource for rice breeding if the mutants have superior eating and/or processing qualities.

Okamoto et al. (2013) screened two non-glutinous upland cultivars, Hiderishirazu-D and Kurnai, and both lacked BEI activity in the developing endosperm. Of these, Kurnai was used for evaluating the effect of BEI deficiency on the hardening of rice cakes, by crossing with the glutinous cv. Naebahata-mochi. The descendent glutinous F_5 lines homologous for the nonfunctional *BEI* allele had a higher ratio of short amylopectin chains and softer texture in the rice cakes (even when stored at 5 °C for 24 h after processing), compared to those homologous for the functional *BEI* allele. This useful property of the *BEI* mutants was also investigated in rice flour bread baking (Aoki et al. 2015). When the rice used for the flour was changed from normal rice to BEI-deficient Hiderishirazu-D or Kurnai, the bread was significantly softer 2 or 3 days after baking.

Nowadays, not a few people avoid eating wheat-based foods, including bread, due to gluten-related disorders, such as wheat allergy and celiac disease (Sapone et al. 2012). Rice flour offers a promising substitute for wheat flour in baking for people so affected. However, a major weakness of rice flour bread is its quicker hardening (Kadan et al. 2001; Yamauchi et al. 2004). Therefore, BEI-deficient rice could be a useful resource for rice breeding; and in fact, several breeding programs aimed at producing commercial glutinous and non-glutinous cultivars with BEI deficiency are currently underway in Japan.

21.5 QTLs Related to the Eating Quality of Improved *japonica* Cultivars

21.5.1 QTL Analyses for High Eating Quality Using Leading Japanese Cultivars

Whole-genome sequencing of rice was accomplished in 2005, and now thousands of re-sequenced rice genome data are available (International Rice Genome Sequencing Project 2005; Li et al. 2014). However, pinpointing the genes responsible for the eating quality traits among improved cultivars, such as the current Japanese cultivars, remains a challenge, because most of them are classified as temperate *japonica* and have narrow genetic variation. The majority has common Wx^b and *alk* alleles regulating AC and AAS, respectively, and the genetic analysis requires highly accurate phenotyping to detect small but commercially important differences in eating qualities.

Here, I will discuss the continued efforts to detect differences in eating quality between Japanese cultivars of the temperate *japonica* type. Several QTL analyses have been conducted using mapping population derived from crossing cultivars with high eating quality and those with low eating quality (Takeuchi et al. 2008; Kobayashi and Tomita 2008; Wada et al. 2008, 2013) . Each study conducted sensory tests for eating quality evaluation, together with physicochemical measurements, and successfully detected several QTLs. Interestingly, all the studies detected a QTL which improved eating quality with Koshihikari genotype or its relative cultivar Sakihikari genotype on the short arm of chromosome 3 with the nearest SSR marker RM4108. This QTL improves the stickiness, glossiness, taste, and overall eating quality of cooked rice without affecting AC. The effect of this QTL on eating quality was further investigated by assaying the genotype of the RM4108 marker with 146 non-glutinous temperate *japonica* cultivars, which consisted of 117 improved and 29 local cultivars (Hori et al. 2016). Among the three detected alleles of RM4108, the A and B alleles were prevalent in modern cultivars with better eating quality (49/58 for the A allele and 61/63 for the B allele), while the C allele appeared with high frequency in the landraces (18/25), of lower eating quality. These results suggest that the QTL close to the RM4108 is quite valuable for improving the eating quality in the modern temperate *japonica* cultivars and, perhaps, is also applicable to improving the eating quality of hybrids between temperate and tropical *japonica* or *indica*, where the consumers prefer glossy and sticky cooked rice.

21.5.2 Fine-Tuning Rice Eating Quality in Japan by Adjusting Amylose Content

Another approach currently employed by Japanese rice breeders is the creation and utilization of low-amylose mutants including natural variations. Roughly 40 - low-amylose cultivars have been officially registered since the first commercial low-amylose cultivar, Aya, was released in 1991. Milky Queen is one of the cultivars screened from an N-methyl-N-nitrosourea-mutagenized population of Koshihikari. It has a slightly dull endosperm phenotype with a low-to-moderate AC of 9–10%. Sato et al. (2002) confirmed that the responsible mutation was in the *Wx* locus, where there were two missense SNPs in exon 4 and 5 of the gene. Later, *Wx* (*GBSSI*) gene sequencing of the cv. Milky Princess, which inherited its low-amylose trait from Ko272, a sibling line of Milky Queen, was performed, and only the SNP in exon 4 was detected (Yang et al. 2013). Since the AC of Milky Princess is similar to that of Milky Queen, the SNP in exon 4, causing an amino acid change from Arg to His, is the critical mutation for the amylose synthesis. This amino acid residue is in the catalytic domain of GBSSI and is conserved among GBSS of major crops such as wheat, barley, maize, potato, and rice. Both of the low-amylose alleles, Wx^{mq} of Milky Queen and Wx^{mp} of Milky Princess, are now utilized in rice breeding in both Japan and China (Takeuchi et al. 2013; Wang et al. 2010).

For the many consumers who prefer slightly sticky rice, cultivars with a low AC of around 10%, such as Milky Queen, are too sticky. In addition, some of low-amylose cultivars, with AC around 5%, tend to produce an unfavorable odor known as "glutinous odor" upon cooking (Fukuda et al. 2014). Therefore, resources that slightly lower the AC are expected. One of the *Wx* alleles, *Wx*1-1, and the low-amylose QTL, *qAC9.3* and *qAC2*, have been selected and utilized for this purpose (Ando et al. 2010; Takemoto-Kuno et al. 2015). *Wx*1-1 of the Hokkai287 is the mutant allele of Wx^{b} induced by cell culture, which has a 37-base deletion in intron 10 of the *Wx* gene. A slightly reduced amount of Wx protein caused by *Wx*1-1 results in a 6–8% decrease in AC. The *qAC9.3* and *qAC2* discovered in the breeding lines are natural variations and slightly decrease AC by 2–3% and 1–2%, respectively. These low-amylose genes are utilized for the fine-tuning of AC, to improve the eating quality, in order to respond to the consumers' preference in Japan.

Information on over 80 QTLs for eating quality, and 50 QTLs for amylose content, has been collected in the Q-TARO and GRAMENE databases (Yonemaru et al. 2010; Monaco et al. 2014). Some of the low-amylose genes, including *du1* and *du3*, have been cloned and characterized (Zeng et al. 2007; Isshiki et al. 2008). The *Wx* alleles and QTLs for low-amylose content mentioned above, and others, can also be selected based on the preferred AC level and the resource background (i.e., *indica*- or *japonica*-based).

21.6 Conclusion

Major differences in the key characteristics of rice quality traits, including AC, GC, and ASS, now can be explained in terms of differences in the SNPs, or insertions or deletions in the responsible genes. The links between amylose and amylopectin LC synthesis by GBSSI have been established, and the correlation between the amylopectin LC ratio and GC has been clarified. Major QTLs regulating the eating and processing quality of rice have been identified, and some of these have been discussed in this review. These include SNPs in intron 1 and exon 10 of the *Wx* gene, with the former closely related to differences in AC between *indica* and *japonica* rice, while the latter is linked to the amylopectin LC ratio and GC. The two SNPs in exon 8 of the *SSIIa* gene cause differences in ASS and affect the gelatinization temperature and the hardening of cooked and processed rice. The increase in short-chain ratio of BEI-deficient rice can maintain the softness of rice-based foods.

As summarized in this review, natural and induced mutations related to starch synthesis have been successfully utilized in rice breeding. However, we have experienced that pyramiding double or triple mutations to create rice with novel property can, in turn, cause reductions in grain size and weight, shrunken and fragile grain, and low fertility, which results in low yield. We need to explore allelic variations in the genes affecting rice eating quality, which can modify or intensify a specific function or mode of action, rather than those that render the enzyme nonfunctional, and contribute to tailor-made breeding. Minor but commercially important QTLs are also becoming major targets to meet the consumers' demand.

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Chapter 22
Genetic Dissection and Breeding for Grain Appearance Quality in Rice

Kiyosumi Hori

Abstract Grain quality largely determines the market price of rice. Many consumers pay particular attention to high grain quality, although preferences in terms of grain size, grain shape, storage components, and fragrance are diverse. Grain chalkiness is one of the most important traits in grain appearance in both *indica* and *japonica* cultivars. Grain chalkiness critically decreases market value because of grain breakage during milling and decreased cooking and eating qualities. Recent progress in the genetic analysis of grain chalkiness has identified many quantitative trait loci (QTLs) and their underlying genes. These results provide insights into the genetic control of grain quality. To reduce grain chalkiness, breeding programs have introduced several QTLs or genes with large genetic effects into the genetic backgrounds of *indica* and *japonica* cultivars. The resultant near-isogenic lines showing high grain quality are good candidates for novel cultivars with improved grain quality.

Keywords Rice · Grain quality · Grain appearance · Chalkiness · High temperature

22.1 Introduction

Rice (*Oryza sativa* L.) is the most important food crop in the world, being a staple for over half of the world's population. Rice cultivars must produce high yields and have strong stress resistance. However, the market price and consumer acceptance of rice are largely determined by rice grain quality, which is a primary consideration of consumers, the food industry, farmers, and seed producers (Hori and Yano 2013; Bao 2014). Therefore, rice grain quality is a major target in rice breeding programs (Champagne et al. 1999; Fitzgerald et al. 2009).

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Rice grain quality comprises a set of complex traits encompassing a wide range of physical, chemical, and physiological characteristics (Hori and Yano 2013). Grain quality consists of four major components, concerning grain appearance, milling qualities, nutrition, and cooking and eating qualities (Bao 2014). This chapter focuses on grain chalkiness, one of the most important traits in grain appearance. Recent progress in the genetic analysis of grain chalkiness has revealed many QTLs and genes, which can be used in breeding selection for the development of novel cultivars with high grain appearance quality.

22.2 Components of Grain Appearance Quality

Pericarp color, grain shape, grain size, and grain chalkiness (translucency) contribute to grain appearance. Several rice cultivars have red, brown, purple, or black pericarps (Wang and Shu 2007). Proanthocyanidins and anthocyanins accumulate in the grains of red and black rice, respectively. Both chemicals have antioxidant activity with health benefits for humans (Maeda et al. 2014). Grain size and shape are described by the kernels' length, width, and thickness. Medium- and short-grain cultivars are generally preferred in Japan, northern China, and Korea, where *japonica* rice is cultivated. Long and slender grain even with aroma cultivars are preferred in many countries where *indica* rice is cultured and traded (Bergman et al. 2004). Previous molecular genetic studies have identified many QTLs and genes involved in the control of grain size and shape and pericarp color (Furukawa et al. 2007; Huang et al. 2013) (See also Chap. 11).

22.3 Grain Chalkiness

Chalky grains have opaque spots in various regions of the endosperm (Hori and Yano 2013; Sreenivasulu et al. 2015; Ishimaru et al. 2016; Lo et al. 2016). Generally, increasing chalkiness reduces grain quality and retail prices. The physicochemical properties involved in grain chalkiness have been investigated for over a hundred years (Inagaki 1899; Goto 1904). Normal translucent endosperm consists of large, tightly packed, polyhedral, single starch granules. On the other hand, chalky endosperm consists of small, loosely packed, round, compound starch granules, and therefore its cause is inferred by the defects in genes affecting starch biosynthesis, starch granule structure, or grain-filling manner (Bao 2014; Lo et al. 2016). In *indica* cultivars, chalkiness is measured visually on a scale of 0–9, where 0, none; 1, minor (<10% of the endosperm); 5, moderate (10–20%); and 9, extensive (>20%) (Fig. 22.1a). In *japonica* cultivars, chalkiness is classified according to the location of the chalkiness: white-back chalkiness on the dorsal side of the grain, white-belly chalkiness on the ventral side, white-base chalkiness around the

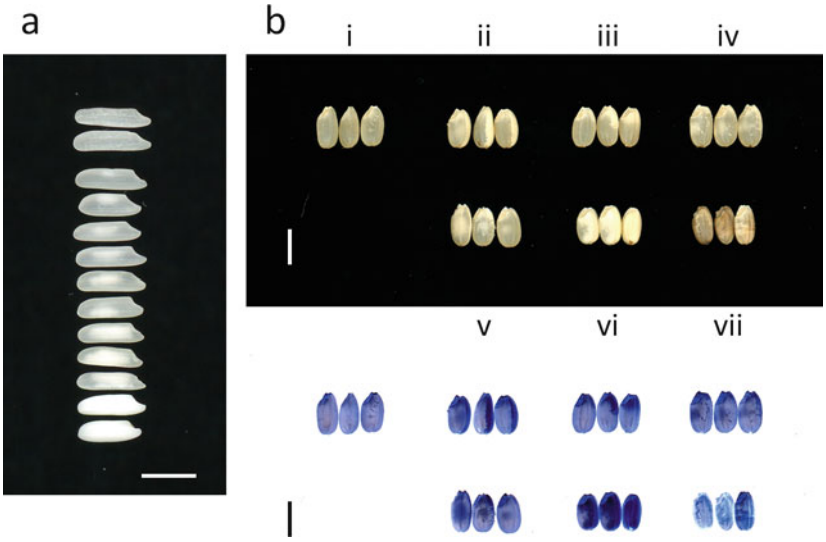

Fig. 22.1 Grain appearance in rice cultivars. (**a**) Chalky grains of *indica* cultivars. (**b**) Chalky grains of *japonica* cultivars: (*i*) normal (no chalkiness), (*ii*) white-back, (*iii*) white-base, (*iv*) white-belly, (*v*) white-core, (*vi*) milky-white, and (*vii*) abortive in normal color (upper) and color-negative (lower) images. Scale bars, 5 mm

embryo, white-core chalkiness in the center, and milky-white chalkiness of the whole grain (Fig. 22.1b).

However, consumer preferences necessitate white-core or whole-grain chalkiness in some *japonica* cultivars (Juliano and Hicks 1996; Hori and Yano 2013). For example, arborio-style and Japanese "sake" brewing cultivars need white-core chalkiness, and glutinous rice cultivars have a completely opaque endosperm. Therefore, genetic control of grain chalkiness is necessary for both increasing and decreasing chalkiness.

22.4 Alteration of Milling Quality by Grain Chalkiness

Chalky grains show significantly different physicochemical, morphological, thermal, cooking, and textural properties from completely translucent grains. In addition, grain chalkiness greatly influences milling quality, especially in *indica* cultivars, which have slender grains (Del Rosario et al. 1968; Bao 2014). Although annual world rice production is 750 Mt a year, the final milled rice yield is 490 Mt a year. Thus, as much as 260 Mt a year is lost after milling, although this includes hulls and bran (Sreenivasulu et al. 2015). Chalky grain is brittle and is easily broken during milling and so decreases the final yield of polished grains.

Grain size and shape are highly correlated with degree of chalkiness and milling quality (Zheng et al. 2007). Grain length is negatively associated with milled-grain yield, whereas grain width and thickness are positively correlated with milled-grain yield. Many QTLs for grain size and shape are co-localized on the same chromosome regions as QTLs for chalkiness (He et al. 1999; Tan et al. 2000; Wan et al. 2005; Ebitani et al. 2008; Wang et al. 2016a). Thus, optimization of grain size and shape would be an effective strategy for decreasing chalkiness and improve milling quality. In particular, because many *indica* cultivars have long and slender grains, the genetic control of grain size and shape in these cultivars needs to be improved to increase milled-grain yield while keeping chalkiness low.

22.5 Effect of High Temperature at Grain-Filling Stage on Grain Chalkiness

Rice growth is seriously affected by climate change, and global warming has become a major constraint on rice production. In the past three decades, Earth's surface temperature has warmed faster than at any time preceding 1850. The global mean surface temperature may rise by up to 4.8 °C by the end of this century relative to the period from 1986 to 2005 (IPCC 2013; Ishimaru et al. 2016). Rice is highly sensitive to heat stress, particularly during the reproductive and grain-filling stages (Mitsui et al. 2013; Jagadish et al. 2015). Heat stress induces abnormal grain formation at all stages from pollen development through endosperm filling to harvest. High temperatures during grain filling reduce starch accumulation and shorten the grain-filling period, resulting in low grain weight and increased chalkiness characterized by both irregular and round starch granules (Arshad et al. 2017).

In *japonica* cultivars, grain chalkiness is induced under conditions of average daily mean temperatures above 27 °C during the first 20 days after heading (Wakamatsu et al. 2009; Ishimaru et al. 2016). High nighttime temperatures significantly increase grain chalkiness. Insolation strongly influences the emergence of grain chalkiness under high temperatures or high humidity (Wakamatsu et al. 2009; Tanaka et al. 2010): the prevalence of white-back grains increases under high insolation and high humidity, while that of milky-white grains increases under low insolation and high humidity. Nitrogen application also influences the frequency of chalkiness (Tanaka et al. 2010): the prevalence of milky-white, white-base, and white-back grains decreased with increased nitrogen uptake and content. This association suggests that maintaining a high nitrogen content at the panicle formation stage is important for optimizing protein content and reducing chalkiness.

Crop management can enhance heat resistance in rice plants (Bita and Gerats 2013; Sreenivasulu et al. 2015). Increasing the water depth in paddy fields is one of the most effective methods to manage heat stress and to minimize damage to

panicles and grains. A water depth of 20–25 cm significantly shields panicles and grains from injury during the grain-filling period. Shifting earlier or later the planting time can also avoid grain filling during hot weather. Such management methods prove successful at reducing grain chalkiness. Collaboration among farmers, breeders, physiologists, and meteorologists is necessary to realize this.

22.6 Genetic Dissection of Grain Chalkiness

Understanding of the genetic factors associated with rice grain quality is necessary for the efficient development of new cultivars producing high-quality grain required and preferred by consumers. Shumiya et al. (1972) reported that the occurrence of white-core grains varied from 0% to 49% in 49 *japonica* cultivars. Ebata and Tashiro (1973) reported that the occurrence of white-belly grains ranged from 0.3% to 99.4% in 88 *indica* and *japonica* cultivars. As the occurrence of chalky grains in F_2 populations showed continuous frequency distributions (Kamijima et al. 1981; Takeda and Saito 1983), grain chalkiness represents a set of complex traits controlled by multiple genes or QTLs.

Many QTLs for grain chalkiness have been detected in segregating populations such as F_2 populations and recombinant inbred lines (RILs) derived from crosses between *indica* and *japonica* cultivars (Yamakawa et al. 2008; Bao 2014; Ishimaru et al. 2016). Genetic studies have detected over 140 QTLs for grain chalkiness across all 12 rice chromosomes in the Gramene QTL database (http://archive.gramene.org/qtl/) (Monaco et al. 2014; Sreenivasulu et al. 2015). He et al. (1999) reported the first detection of QTLs for grain chalkiness, detecting three QTLs for the percentage of white-core grains and the square of the white-core in doubled haploid lines (DHLs) of ZYQ8 × JX17. Tan et al. (2000) found 14 QTLs for white-belly, white-back, and white-core grains in RILs of Zhenshan 97 × Minghui 63. Ebitani et al. (2008) reported 12 QTLs for 6 types of grain chalkiness in chromosome segment substitution lines (CSSLs) of Nipponbare × Koshihikari.

A few QTLs for grain chalkiness have so far been fine mapped and isolated. *qPGWC8* is a major-genetic-effect QTL for this phenotype on chromosome 8 in CSSLs of Asominori × IR24 (Wan et al. 2005). Guo et al. (2011) narrowed down its chromosomal region within a 142-kbp region by using 1801 BC_4F_2 plants of Asominori × IR24. *qPGWC7*, on chromosome 7, was identified using a set of CSSLs of PA64s × 9311: fine mapping in a population of 3221 F_2 plants delimited it to a 44-kbp region including 13 predicted genes (Zhou et al. 2009). qACE9 was also detected in CSSLs of PA64s × 9311 (Gao et al. 2016): fine mapping in 920 BC_4F_2 plants narrowed it down to within a 22-kbp region including five predicted genes. Map-based cloning of the *chalkiness 5* (*CHALK5*) QTL, a major-effect QTL in DHLs of H94 × Zhenshan 97, isolated a gene encoding a vacuolar H^+-translocating pyrophosphatase with activity of inorganic pyrophosphate hydrolysis and H^+-translocation (Li et al. 2014) (Table 22.1).

Table 22.1 Grain chalkiness genes in rice isolated by map-based cloning strategies

Gene name	Synonym	Chr	RAP ID[a]	MSU ID[b]	Description	References
Starch biosynthesis and metabolism pathway genes						
SSG4		1	Os01g0179400	LOC_Os01g08420	Amyloplast-localized protein containing DUF490, regulation of starch grain sizes	Matsushima et al. (2014)
GIF2	*OsAGPL2*	1	Os01g0633100	LOC_Os01g44220	ADP-glucose pyrophosphorylase large subunit, control of starch biosynthesis in endosperm development	Tang et al. (2016) and Wei et al. (2017)
OsBT1		2	Os02g0202400	LOC_Os02g10800	ADP-glucose transporter, plastidic translocator, starch synthesis during seed development	Cakir et al. (2016) and Li et al. (2017)
SBEIIb	*SBE3*	2	Os02g0528200	LOC_Os02g32660	Starch branching enzyme 3, starch synthesis	Butardo et al. (2011) and Yang et al. (2012)
Amy1A		2	Os02g0765600	LOC_Os02g52710	Alpha-amylase glycoprotein, degradation of starch granules	Hakata et al. (2012)
PSR1		5	Os05g0121600	LOC_Os05g03040	AP2/EREBP family transcription factor, starch biosynthesis	Fu and Xue (2010)
OsbZIP58		7	Os07g0182000	LOC_Os07g08420	Basic leucine zipper transcriptional activator, grain filling	Wang et al. (2013)
FLO4	*OsPPDKB*	5	Os05g0405000	LOC_Os05g33570	Orthophosphate dikinase precursor	Kang et al. (2005)
PFP1		6	Os06g0247500	LOC_Os06g13810	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase (PFP) beta subunit, regulation of carbon metabolism during grain filling	Duan et al. (2016)
SSIIIa	*FLO5*	8	Os08g0191433	LOC_Os08g09230	Starch synthase, starch biosynthesis	Ryoo et al. (2007) and Fujita et al. (2007)

Amy3E		8	Os08g0473600	LOC_Os08g36900	Alpha-amylase isozyme 3E precursor (EC 3.2.1.1), 1,4-alpha-D-glucan glucanohydrolase)	Hakata et al. (2012)
UGPase1		9	Os09g0553200	LOC_Os09g38030	UDPase, pollen mother cell (PMC) meiosis, pollen development	Woo et al. (2008)
Seed storage protein biosynthesis genes						
GLUP2	*GPA4*, *GOT1B*	3	Os03g0209400	LOC_Os03g11100	Vesicle transport protein, regulation of protein export from ER in developing endosperm cells	Wang et al. (2016b) and Fukuda et al. (2016)
GLUP6	*GPA2*, *OsVPS9A*	3	Os03g0262900	LOC_Os03g15650	Activator of Rab5 GTPase, intracellular transport of proglutelin	Fukuda et al. (2013) and Liu et al. (2013)
GPA3		3	Os03g0835800	LOC_Os03g61950	Galactose oxidase/kelch, beta-propeller domain-containing protein	Ren et al. (2014)
ESP2	*PDIL1-1*	11	Os11g0199200	LOC_Os11g09280	Protein disulfide isomerase-like enzyme, starch synthesis, maturation of proglutelin in endosperm	Takemoto et al. (2002)
GLUP4	*OsRab5a*	12	Os12g0631100	LOC_Os12g43550	Small GTPase, storage protein trafficking	Fukuda et al. (2011)
Other grain chalkiness genes						
OsNF-YB1		2	Os02g0725900	LOC_Os02g49410	Component of the NF-Y/HAP transcription factor complex, regulation of endosperm development	Xu et al. (2016)
FLO6		3	Os03g0686900	LOC_Os03g48170	CBM48 domain-containing protein, compound granule formation and starch synthesis	Peng et al. (2014)
GIF1		4	Os04g0413500	LOC_Os04g33740	Cell-wall invertase, carbon partitioning during early grain filling	Wang et al. (2008)
FLO2	*FLO(a)*, *OsTPR*	4	Os04g0645100	LOC_Os04g55230	Tetratricopeptide repeat (TPR) domain-containing protein, grain size and starch quality	She et al. (2010)

(continued)

Table 22.1 (continued)

Gene name	Synonym	Chr	RAP ID[a]	MSU ID[b]	Description	References
CHALK5		5	Os05g0156900	LOC_Os05g06480	Vacuolar H^+-translocating pyrophosphatase, regulation of grain chalkiness	Li et al. (2014)
SSG6	*OsACS6*	6	Os06g0130400	LOC_Os06g03990	ACC synthase, protein homologous to aminotransferase, ethylene biosynthesis, control of starch grain size in rice endosperm	Matsushima et al. (2016)
OsAlaAT1		10	Os10g0390500	LOC_Os10g25130	Alanine aminotransferase, starch synthesis in developing seeds	Yang et al. (2015)
FLO7		10	Os10g0463800	LOC_Os10g32680	Domain of unknown function, DUF1338, containing green-plant-unique protein, regulation of starch synthesis and amyloplast development, peripheral endosperm development	Zhang et al. (2016)

[a]Locus ID of the Rice Annotation Project (http://rapdb.dna.affrc.go.jp/)
[b]Locus ID of the Rice Genome Annotation Project, Michigan State University (http://rice.plantbiology.msu.edu/)

Several rice mutants show grain chalkiness phenotypes due to disruption of genes associated with starch biosynthesis and metabolism pathways (Table 22.1). *Tos17* transposon and T-DNA insertion mutants of *starch synthase IIIa* (*SSIIIa*) showed loose starch granule packing and a chalky phenotype (Fujita et al. 2007; Ryoo et al. 2007). A single nucleotide substitution at the splice junction of *UDP-glucose pyrophosphorylase 1* (*UGPase1*) induced chalky endosperm (Woo et al. 2008); overexpression of UGPase1 protein produced normal grains in transgenic plants of the *UDPase1* mutant. A gene named *floury endosperm 4* (*flo4*) was isolated from a mutant with a floury white-core endosperm caused by T-DNA insertion into the *rice pyruvate orthophosphate dikinase B* (*OsPPDKB*) gene (Kang et al. 2005). Chalkiness was produced by downregulation of *starch-branching enzyme IIb* (*SBEIIb*) in rice endosperm (Butardo et al. 2011; Yang et al. 2012). Likewise, a mutant of the *substandard starch grain 4* (*SSG4*) gene had altered starch granules and a chalky phenotype (Matsushima et al. 2014). Recently, Tang et al. (2016) and Wei et al. (2017) identified *grain incomplete filling 2* (*gif2*), which encodes the rice ADP-glucose pyrophosphorylase large subunit 2 (OsAGPL2) protein. The *rice brittle 1* (*OsBT1*) gene encodes an ADP-glucose transporter, and a mutant had white-core grains (Li et al. 2017). The ethyl methane sulfonate (EMS) mutants of *pyrophosphate: the fructose-6-phosphate 1-phosphotransferase* (*PFP1*) gene exhibited floury endosperm (Duan et al. 2016). Alteration of the expression of starch metabolism genes also influences the degree of chalkiness: downregulation of α-amylase genes *Amy1A* and *Amy3E* produced less chalky grains in RNAi transgenic plants (Hakata et al. 2012). Fu and Xue (2010) reported that *rice starch regulator 1* (*RSR1*) gene, which encodes an APETALA2/ethylene-responsive element binding protein family transcription factor protein, negatively regulates the expression of starch synthesis genes. And *OsbZIP58* is a key transcriptional regulator required for starch synthesis through directly binding to the promoters of *OsAGPL3*, *GBSSI*, *OsSSIIa*, *SBE1*, *OsBEIIb*, and *ISA2* to promote their expression (Wang et al. 2013).

The formation of chalky grain is also triggered by abnormal accumulation of seed storage protein. Many storage protein mutants preferentially accumulate a large amount of glutelin precursor within chalky grains (Table 22.1). The *endosperm storage protein mutant 2* (*esp2*) gene encodes disulfide isomerase-like 1-1 (PDIL1-1) protein (Takemoto et al. 2002) (Fig. 22.2). The *glutelin precursor mutant 4* (*glup4*) gene encodes a rice ortholog of the small GTP-binding and GTP-hydrolyzing protein Rab5a (Fukuda et al. 2011). The *glutelin precursor mutant 6* (*glup6*) gene encodes guanine nucleotide exchange factor, which activates Rab5a protein (Fukuda et al. 2013). The *glutelin precursor accumulation 3* (*gpa3*) gene encodes a plant-specific kelch-repeat protein, which is associated with the trans-Golgi network (Ren et al. 2014). The *glutelin precursor mutant 2* (*glup2*) gene encodes Golgi transport 1B (GOT1B) protein, which has membrane-spanning domains (Fukuda et al. 2016; Wang et al. 2016b). These results indicate that disruption of the storage protein trafficking system during endosperm development elevates the amount of small-vesicle-like abnormal protein bodies, decreases the number and size of protein bodies, and ultimately increases grain chalkiness.

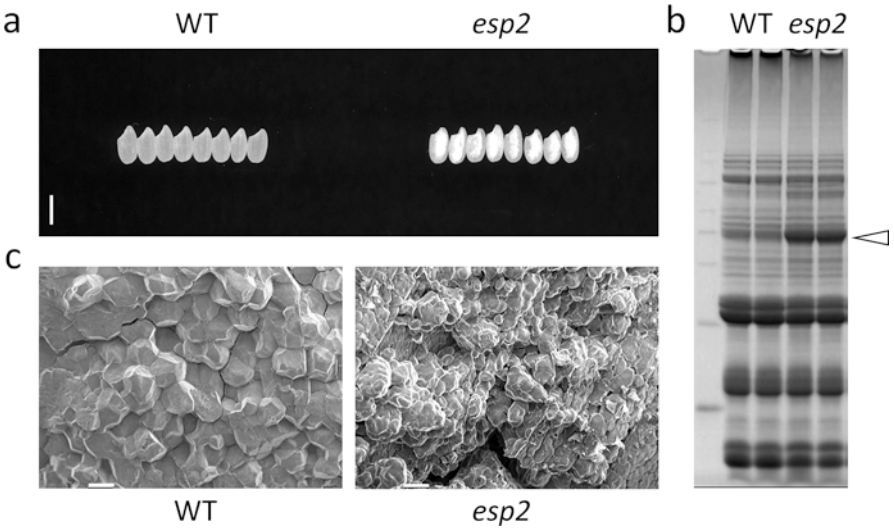

Fig. 22.2 Phenotypes of rice grains in wild type (WT) and *esp2* mutant. (**a**) Appearance of polished rice grains. Scale bar, 5 mm. (**b**) SDS-PAGE image of storage proteins in mature seeds. Arrow indicates accumulation of glutelin precursor in the *esp2* mutant. (**c**) Scanning electron microscopy images of transverse sections of mature seed. Scale bars, 10 μm

Mutations of genes not involved in starch biosynthesis and metabolism or protein biosynthesis can also lead to phenotypes similar to grain chalkiness (Table 22.1). The *grain incomplete filling 1* (*gif1*) mutant has a disrupted allele of a gene encoding a cell-wall invertase protein required for carbon partitioning during early grain filling (Wang et al. 2008). The *floury endosperm 2* (*flo2*) mutation is caused by a disrupted allele of a gene encoding the rice tetratricopeptide repeat domain-containing (OsTPR) protein (She et al. 2010). The *floury endosperm 6* (*FLO6*) gene encodes an unknown protein with a C-terminal carbohydrate-binding module (Peng et al. 2014). The *substandard starch grain 6* (*ssg6*) gene encodes a protein homologous to aminotransferase (Matsushima et al. 2016). The mutation of *OsAlaAT1* gene, which encodes cytosolic aminotransferase associated with the interconversion of pyruvate to alanine, decreased the expression of starch biosynthetic genes (Yang et al. 2015). The *floury endosperm7* (*FLO7*) gene encodes a protein of unknown function; however, *FLO7* is necessary for starch synthesis and amyloplast development (Zhang *et al*. 2016). RNAi knockdown of the expression of the *rice nuclear factor Y B* (*OsNF-YB*) transcription factor gene, which is co-expressed with starch biosynthesis genes in rice endosperm, leads to small grains with chalky endosperm (Xu et al. 2016).

A comprehensive atlas of transcriptomic changes under high temperatures reveals downregulation of starch biosynthesis and upregulation of starch degradation (Jagadish et al. 2015; Yamakawa et al. 2007). The incidence of chalkiness is increased as a result of high expression of genes for starch degradation enzymes such as α-amylases (*Amy1A*, *Amy1C*, *Amy3A*, *Amy3D*, and *Amy3E*; Yamakawa

et al. 2007, Hakata et al. 2012). Heat stress also downregulated the expression of starch synthesis genes of *SBEIIb* and *OsPPDKB* (Li et al. 2011). To decrease grain chalkiness, it would be effective to adjust the balance of gene expression between biosynthesis and metabolism of starch and storage proteins.

22.7 Improvement of Grain Quality in Breeding Selection of Rice Cultivars

DNA markers tightly linked with grain chalkiness QTLs and genes facilitate marker-assisted selection for the development of novel cultivars with appearance of high grain quality. Hori et al. (2012) found two QTLs for grain chalkiness on chromosomes 8 and 11, *qDWK8* and *qDWK11*. Two CSSLs with Koshihikari alleles of these QTLs in the Nipponbare background had lower proportions of white-base and white-back grains (Hori et al. 2017). In addition, CSSL SL844, with *qDWK11*, had fewer abortive and cracked grains after milling (Fig. 22.3). Kobayashi et al. (2007) detected a major-effect QTL for white-back grains on chromosome 6 (*qWB6*). A near-isogenic line (NIL) carrying the Hanaechizen *qWB6* allele of this QTL in the Niigatawase background had significantly less white-back chalkiness during grain filling at >27 °C (Kobayashi et al. 2013). Shirasawa et al. (2013) detected another QTL on chromosome 6 very close to *qWB6*. NILs with the Kokoromachi allele of this QTL in the Tohoku 168 background had fewer white-back grains. Two NILs carrying a QTL on chromosome 8 from Chikushi 52 in the Tsukushiroman background had less white-back chalkiness (Wada et al. 2015). Murata et al. (2014) detected an appearance quality

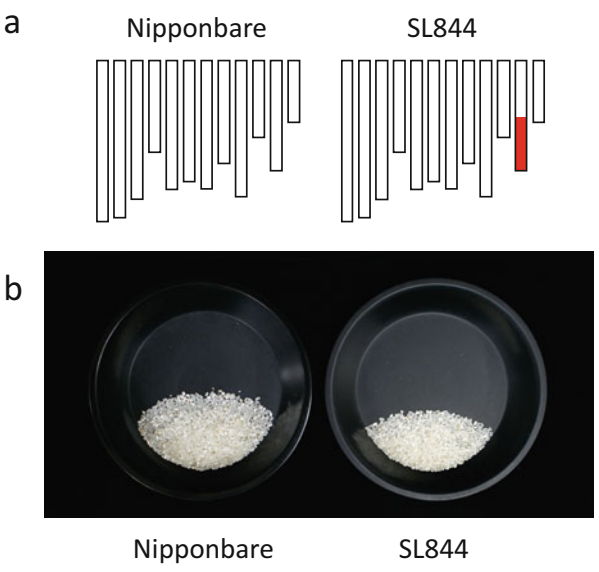

Fig. 22.3 Milling quality of Nipponbare and SL844, with a Koshihikari chromosome fragment (red) in the Nipponbare genetic background (white). (**a**) Graphical genotypes representing the 12 rice chromosomes. (**b**) Abortive and cracked grains after milling 1.5 kg of brown rice

QTL *Apq1* on chromosome 7. A NIL with the Habataki *Apq1* allele in the Koshihikari background had a significantly higher rate of translucent grains. Kobayashi et al. (2016) reported that a NIL carrying the Kasalath allele of the seed dormancy QTL *Sdr4* in the Koshihikari background had a lower rate of white-back grains. Although these reports concern white-back chalkiness mostly, they clearly indicate that introgression of these QTLs can significantly decrease grain chalkiness. These NILs are good candidates for novel cultivars with high grain quality.

It is also important to find additional QTLs and to develop novel NILs by screening many cultivars for new genetic variation. Rice cultivars and wild relatives have a broad range of adaptations to heat stress and can be used in breeding to reduce grain chalkiness (Shumiya et al. 1972; Ebata and Tashiro 1973; Arshad *et al.* 2017). The *indica* cultivars Takanari, Habataki, and Kasalath have low chalkiness (Ebitani et al. 2008; Murata et al. 2014). Several *japonica* cultivars, including Fusaotome, Koshijiwase, Hanaechizen, Kokoromachi, and Tentakaku, have low chalkiness (Kobayashi et al. 2007; Ishimaru et al. 2016). Thus, both *indica* and *japonica* cultivars could be used as donors for reducing grain chalkiness. The use of additional genetic resources would provide advantages in the development of novel rice cultivars with high grain quality.

22.8 Conclusions and Prospects

Many QTLs for grain chalkiness have been detected on all rice chromosomes. Several have been fine mapped, and the responsible genes have been identified by map-based cloning strategies. Further work has led to the development of NILs by marker-assisted selection with DNA markers linked to major-effect QTLs and genes.

However, we are still far from clearly understanding how grain chalkiness forms. To uncover all genetic factors involved in its control, it is necessary to perform further analyses with new techniques. So far, QTL analysis has detected a few QTLs associated with phenotypic differences between pairs of cultivars. Genome-wide association study (GWAS) can collect many QTLs and identify the genes responsible directly without the need for segregating populations (Zhao et al. 2011; Yano et al. 2016). GWAS has recently been used in the study of grain quality traits in rice (Qiu et al. 2016; Wang et al. 2016a). GWAS will be a powerful tool to collect additional QTLs and to isolate new genes in multiple cultivars. Further, novel analytical methods such as genome sequencing by next-generation sequencer, genotype-by-sequencing (GBS), RNA-seq, and metabolome analysis can enhance the resolution of genetic analysis. Chen et al. (2016) combined GBS and RNA-seq to detect grain chalkiness QTLs and to identify candidate genes highly expressed in chalky endosperm. Metabolome analyses in rice cultivars and mutants revealed that the metabolism of carbon, nitrogen, and phospholipids is important in the formation of chalky grains (Yamakawa and Hakata 2010; Kusano et al. 2012; Lin et al. 2017). Such trials could open an opportunity to identify in detail the molecular mechanisms that regulate grain chalkiness.

In addition to the development of NILs carrying single major-effect QTLs and genes, it is possible to develop NILs by introducing multiple QTLs and genes simultaneously. Combining mutant alleles of starch synthase and branching enzyme genes altered amylose content and the chain-length distribution of amylopectin (Fujita et al. 2011; Zhang et al. 2011; Abe et al. 2014; Toyosawa et al. 2016). Such double-mutant lines could reveal how starch biosynthesis genes interact with each other and with the interrelationship between starches and storage proteins and phospholipids. NILs with multiple QTLs and genes would also be more effective at developing higher-grain-quality cultivars than NILs with single ones. For instance, combining a grain chalkiness QTL and a functional impaired allele of *granule-bound starch synthase I* (*GBSSI*) gene could produce and generate novel rice cultivars with low grain chalkiness and high eating quality.

Because the development of grain chalkiness is complex and is determined by multiple QTLs and genes, the development of new cultivars with high grain quality might be challenging for traditional breeding approaches. Other breeding concepts would be more appropriate for improving grain quality controlled by multiple QTLs with small genetic effects. Breeding by genomic selection can estimate the genetic effects (breeding value) of each locus on the whole genome by simultaneously accounting for all SNP markers (Meuwissen et al. 2001). Although genomic selection has been used mainly in dairy cattle breeding (Schaeffer 2006), methods that consider multiple genetic loci simultaneously offer powerful tools to reduce grain chalkiness in the development of novel rice cultivars.

Recent studies have identified many QTLs and genes involved in grain chalkiness, but many more loci are needed for the improvement of current rice cultivars. Advances in research are still needed to reveal the molecular mechanisms of the development of grain chalkiness in rice.

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Chapter 23
Rice Epigenomes: Characteristics, Regulatory Functions, and Reprogramming Mechanisms

Yongfeng Hu and Dao-Xiu Zhou

Abstract Rice is one of the most important food crops in the world and has been established as a model for plant genomic and epigenomic study. In recent years accumulated data by high-throughput sequencing provide useful information on rice epigenomic characteristics. Exploring the function of various epigenetic regulators including writer, reader, and eraser of epigenomic information reveals the importance of epigenomic characteristics to genome activity. It has been also found that these regulators are involved in a diverse range of developmental and stress-responsive pathways. Analysis of different rice varieties indicates that some phenotypic differences are caused by epigenetic variations rather than DNA sequence mutations, which are referred to as epialleles. Significantly, several epialleles are identified to be related to important agronomic traits, which provide novel strategies to improve grain productivity in rice. In this chapter, we review features of rice epigenome, epigenetic regulation of gene expression, and its implication in rice development, stress response, agronomic traits, and yield.

Keywords Rice · Epigenome · Regulation · Development · Stress

23.1 Characteristics of Rice Epigenomes

Epigenetics that studies maintenance, resetting, and inheritance mechanisms of gene expression states during cell division and differentiation is the basis for plant development and adaptation to the changing environment. Dynamic modification of chromatin structure is the basis of epigenetic regulation of gene

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expression, DNA replication and repair, and RNA splicing. Nucleosome that constitutes of an octamer of core histones and a segment of about 147 bp of DNA is the basic structure of chromatin. The histone octamer comprises two copies of H3, H4, H2A, and H2B. Chromatin modifications include DNA methylation, histone modifications, histone variant deposition, nucleosome positioning, etc. Epigenomes are genome-wide chromatin modification profiles established in specific cell types/tissues or under specific growth conditions. In the past years, genome-wide DNA methylation and several types of histone modifications have been studied in several rice tissues. Epigenomic variations that alter transcriptomic profiles have consequences in variations of phenotypes and agronomic traits. Study of a number of rice chromatin modification enzymes uncovers their important functions in epigenetic regulation, gene expression, plant development, and adaptation to adverse growth conditions.

23.1.1 DNA Methylation

DNA methylation is a process by which methyl groups are transferred to cytosine of DNA from donors. Extensive data reveal conserved DNA cytosine methylation in plants including rice, which occurs in CG, CHG, and CHH sequence contexts (H is A, C, or T) (Law and Jacobsen 2010). In *Arabidopsis*, de novo cytosine methylation in all sequence contexts is carried out by CHROMOMETHYLASE2 (CMT2) and DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2), while maintenance of CG, CHG, and CHH methylation is mediated by METHYLTRANSFERASE1 (MET1), CMT3, and DRM2, respectively(Lindroth et al. 2001; Saze et al. 2003; Stroud et al. 2014). CMT2 is recently shown to also maintain CHG and CHH methylation (Stroud et al. 2014). In rice, two *MET1* homologs have been identified: *MET1a* and *MET1b (*Teerawanichpan et al. 2004*;* Yamauchi et al. 2008*)*. It seems that *MET1b* is more important as higher expression levels of *MET1b* than *MET1a* were observed, and loss of function of *MET1b* leads to genome-wide reduction of CG methylation (Yamauchi et al. 2008, 2014; Hu et al. 2014). In addition, null mutation of *MET1b* caused seedling lethality, suggesting the important role of the gene in rice normal development. Function of *OsDRM2* in rice is likely to be different from that in *Arabidopsis*. In vitro assay demonstrates that it has de novo DNA methyltransferase activity in all sequence contexts (Pang et al. 2013; Sharma et al. 2009). DNA demethylation is catalyzed by DNA glycosylases, which are encoded by six genes in the rice genome (Zemach et al. 2010a). Among them, only DNG701, apparently an ortholog of *Arabidopsis* ROS1, has been proved to be responsible for the demethylation of *Tos17*, a Ty1-copia retrotransposon, which is important for *Tos17* transposition (La et al. 2011). In addition, there seems no rice ortholog of *Arabidopsis* DEMETER that is specifically involved in genome-wide demethylation process in the central cells. This suggests that there may be a different mechanism for DNA methylation reprogramming during female gametogenesis in rice.

Amounts of genome-wide DNA methylation data have been generated in rice (Zemach et al. 2010a, b; Chodavarapu et al. 2012; Feng et al. 2010; He et al. 2010; Li et al. 2012; Yan et al. 2010; Tan et al. 2016). Basically, DNA methylation patterns in rice resemble those in *Arabidopsis thaliana*. However, the rice genome displays a different DNA methylation landscape and has a much higher level of genome-wide DNA methylation (Li et al. 2012). The features of DNA methylation landscapes in rice are as followings:

1. The methylation levels of CG, CHG, and CHH and total cytosine methylation level in rice genome are much higher than those in *Arabidopsis* (Li et al. 2012).
2. CG and CHG methylations are centered in heterochromatic region enriching transposable elements and related genes, whereas CHH methylation is distributed over the short transposable elements (less than 1 kb) in euchromatic region (Tan et al. 2016). There are plenty of MITE class short DNA transposable elements in rice genome. These transposable elements are located to the intergenic regions. Their distribution is well overlapped with that of genome-wide CHH methylation (Zemach et al. 2010a; Tan et al. 2016). Recent study indicates that CHH methylation on MITE plays an important role in the regulation of adjacent genes (Tan et al. 2016; Wei et al. 2014).
3. CHH and CHG methylations have also been found in functional genes. Loss of CHH or CHG methylation results in activation of many genes. This demonstrates that CHH and CHG methylations are directly involved in gene repression (Tan et al. 2016).
4. CG methylation levels are stable across different tissues or organs, while non-CG methylation is increased with plant growth and aging (Zemach et al. 2010a). In rice endosperm, non-CG methylation is found to be globally decreased across the genome, whereas CG hypomethylation is localized (Zemach et al. 2010a).
5. The DNA associated with all the DNase I hypersensitive (DH) sites in seedlings is generally hypomethylated (Zhang et al. 2012a). However, tissue-specific DH sites located in the promoters showed higher levels of DNA methylation than the average level associated with all DH sites located in promoters in seedlings (Zhang et al. 2012a). It was hypothesized that the tissue-specific DH sites may represent poised DH sites facilitating DNA demethylation to fully activate gene expression (Zhang et al. 2012a; Chen and Zhou 2013).

23.1.2 Histone Modification

Histone modifications on lysine and arginine residues occur mainly at N-terminal tails of histones, which extend out of nucleosome core particles. Histone acetylation refers to addition of acetyl group to lysine residues of histone. Histone lysine residues can be mono-, di-, or tri-methylated, and histone arginine residues are found to be mono-methylated, asymmetric, and symmetric di-methylated. By immunoprecipitation and western blot analysis, many histone lysine modification

sites have been identified in rice. Some of them can be either acetylated or methylated (e.g., H3K4, H3K9, H3K27, and H3K36), and rest of them can only be acetylated (e.g., H3K14, H3K18, H3K23, H4K5, H4K8, H4K12, and H4K16) (Ding et al. 2012; Du et al. 2013; Lu et al. 2015; Mahrez et al. 2016; Roy et al. 2014; Tan et al. 2011; Yin et al. 2008; Liu et al. 2015, 2016).

Histone lysine is acetylated by histone acetyltransferase (HAT). Rice has eight HATs, whose function has not been analyzed yet, except OsGCN5, GENERAL CONTROL NON-REPRESSIBLE 5 (Liu et al. 2012). Our recent paper demonstrates that OsGCN5 could acetylate H3 and H4 as its homolog in *Saccharomyces cerevisiae* and mammals (Zhou et al. 2017). Histone lysine deacetylation is catalyzed by histone deacetylase (HDAC). Two of 19 rice HDACs have been characterized to have deacetylation activity. OsSRT1, a SILENT INFORMATION REGULATOR2 (SIR2) family protein, which removes H3K9ac and HDT701/OsHDT1 is a H4 deacetylase (Ding et al. 2012; Huang et al. 2007; Li et al. 2011a).

Histone lysine is methylated by Su(Var)3-9, Enhancer-of-Zeste, and Trithorax (SET)-domain proteins, which is grouped into several families based on their sequence similarity. Members in different families catalyze histone lysine methylation at different sites (Zhou and Hu 2010). For example, SUVH, SU(VAR)3-9 homolog members SDG714 and SDG728, are involved in H3K9 methylation (Qin et al. 2010). Enhancer-of-Zeste (E(Z)) homologous proteins SDG711 and SDG718 are responsible for H3K27me3 methylation in rice (Liu et al. 2015). ASHH, ASH1 homolog family members of SET-domain proteins SDG725, SDG724, and SDG708, are rice H3K36 methyltransferases (Liu et al. 2016; Sui et al. 2012; Sun et al. 2012). Although trithorax homolog (TXH) family member SDG723 has weak histone H3 methyltransferase activity in vitro, it is not clear whether it specifically catalyzes H3K4 methylation as its homolog in *Arabidopsis (*Choi et al. 2014*)*. Histone lysine methylation can be removed by two classes of histone demethylase: lysine-specific demethylase 1 (LSD1) and Jumonji C (JmjC) domain-containing proteins (Shi et al. 2004; Tsukada et al. 2006). Function of LSD1 homologs in rice has not been characterized yet. Catalytic function of 4 of 20 JmjC domain-containing proteins in rice has been analyzed. JMJ706 catalyzes H3K9 demethylation (Sun and Zhou 2008). JMJ705 specifically demethylases H3K27me2/3 (Li et al. 2013). JMJ701, JMJ703, and JMJ704 remove H3K4 methylation (Chen et al. 2013; Cui et al. 2013; Hou et al. 2015; Yokoo et al. 2014). H3K36me3 demethylase has not been identified in rice.

In rice the direct evidence for histone arginine methylation has not been provided. Protein arginine methyltransferases (PRMTs), which show specific methyltransferase activity, have been characterized. Eight PRMTs identified in rice are classified into Type I and Type II based on the formation of type of dimethylarginine (Ahmad et al. 2011). For example, OsPRMT1 asymmetrically methylates H3 at R17 and H4 at R3. OsPRMT10 asymmetrically methylates H3 at R2 and H4 at R3. OsPRMT4 methylates H3R17 and OsPRMT6b asymmetrically methylates H3 at R2. These enzymes belong to Type I PRMT. OsPRMT5 symmetrically methylates R3 in histones H4 and thus is a Type II PRMT (Ahmad et al. 2011).

Although many histone modification sites have been identified in rice, genome-wide distribution of only part of the modifications has been analyzed (e.g., H3K4me2, H3K4me3, H3K9ac, H3K23ac, H3K27ac, H3K27me3, H3K36me1, H3K36me2, H3K36me3, H4K12ac, and H4K16ac (He et al. 2010; Zhang et al. 2012a; Du et al. 2013; Lu et al. 2015; Liu et al. 2016; Zhou et al. 2016). Some of the modifications show similar distribution patterns. For instance, H3K4me3, H3K9ac, and H3K27ac are mainly enriched at the 5′ end of genes downstream of the transcription start sites (TSSs) (Du et al. 2013). However, H3K27ac enrichment is highest in intergenic regions compared to the other histone modifications, suggesting that H3K27ac also marks active enhancers (Du et al. 2013). Compared to *Arabidopsis*, rice H3K23ac and H4K16ac are more enriched in the gene body (Lu et al. 2015). The two marks are mainly found in the coding regions of lowly expressed genes. However, genes enriched with both marks show higher expression levels than genes enriched with only one mark or with neither mark (Lu et al. 2015). The distribution patterns of H3K36me1, H3K36me2, and H3K36me3 in genic regions are distinct from each other (Liu et al. 2016). H3K36me2 is most abundant near transcription termination site (TTS), whereas H3K36me3 is most abundant near TSS. For H3K36me1, two peaks are present upstream of TSS and downstream of TTS, respectively (Liu et al. 2016). In contrast, although enriched in genic region, H3K27me3 is distributed all over the gene body with no obvious peak toward 5′ or 3′ end (He et al. 2010). H3K4me3 and H3K27me3 are mutually repulsive and antagonist marks for gene expression in plants (Hu et al. 2012; Zhang et al. 2009). However, high concurrence of H3K27me3 and H3K4me2 suggests that two marks are involved in priming stress-responsive or developmental gene activation in rice (Chen and Zhou 2013; Hu et al. 2012).

Recently it is shown that many H3K27me3-marked developmental genes were also methylated at non-CG (CHG and CHH) sites in the body regions of rice genes (Zhou et al. 2016). SDG711 physically interacted with OsDRM2 (CHH methyltransferase) and a SRA domain (capable of binding methylated CHG sites) -containing protein SDG703. Mutation of *OsDRM2* resulted in loss of H3K27me3 from many genes (Zhou et al. 2016). These results together suggest that the repression of many developmental genes may involve both DRM2-mediated non-CG methylation and SDG711-mediated H3K27me3 and that the two marks are not generally mutually exclusive but may cooperate in repression of developmentally regulated genes in rice (Zhou et al. 2016). This point seems to be different from what is known in *Arabidopsis* in which H3K27me3 and non-CG methylation are mutually exclusive (Lindroth et al. 2008; Weinhofer et al. 2010). This difference may be due to extensive differences of genomic structures of the two species. It remains to know whether the two marks are mutually involved in the maintenance of each other during cell division in rice.

23.1.3 Histone Variant

Histone variants are series of histones with different amino acid sequence compared to canonical histones. The difference could be a few amino acids or a long peptide segment. Canonical histones are incorporated into nucleosome during S phase after DNA replication, whereas histone variants could be deposited in a DNA replication-independent manner. H2A.Z and H3.3 have been proved to be important transcription regulators (Weber and Henikoff 2014). In rice three H2A.Z genes and three H3.3 genes have been identified, respectively (Hu and Lai 2015). Three H3.3 proteins show identical amino acid sequence and differ by four amino acids with canonical H3. H2A.Z protein sequence is also conserved with longer N-terminal than canonical H2A.Z (Hu and Lai 2015). Genome-wide distribution of H2A.Z in rice different tissues has been analyzed recently. The results demonstrate that H2A.Z is mainly enriched across gene body (Zhang et al. 2017a). H2A.Z enrichment peaks were also observed at 5′ and 3′ end of highly expressed genes, which are similar to the H2A.Z distribution pattern in *Arabidopsis* (Zhang et al. 2017a*;* Coleman-Derr and Zilberman 2012).

23.2 Epigenetic Regulation of Gene Expression

Although the mechanism how chromatin modifications regulate gene expression has not been fully elucidated, correlation between chromatin modification marks and gene transcription activity is clear. Histone acetylation, H3K4me3, and H3K36me3 correlate with actively transcribed genes, whereas DNA methylation and H3K27me3 are enriched in silent genes. Related studies were also carried out in rice. The results show that gene expression correlates with relevant chromatin modification changes. For example, when submergence induced *alcohol dehydrogenase 1* (*ADH1*) and *pyruvate decarboxylase 1* (*PDC1)* expression, levels of H3K4me3 and H3 acetylation on the loci were increased. After reaeration, ADH1 and PDC1 mRNA levels were decreased, and H3K4me3 and H3 acetylation dropped to initial state (Tsuji et al. 2006). However, H3K4me3 and H3 acetylation may play different roles in the induction of the two genes as H3K4me3 level was increased earlier than H3 acetylation (Tsuji et al. 2006). Similarly, jasmonic acid-induced stress response gene expression results in H3K27me3 removal from the genes, which is dependent on the histone demethylase JMJ705 (Li et al. 2013). There are also many developmental genes revealed to be regulated epigenetically, which will be described in the next section.

It was proposed that histone modification interferes with association between histone and DNA thus facilitating gene transcription. However, this proposition could not explain that histone methylation either positively or negatively regulates gene expression. Now it is generally accepted that histone modification serves as recognition marks bound by specific proteins also called effectors or readers.

Several domains have been identified to bind specific histone modification marks such as chromodomain, Tudor, PWWP, WD40, PHD domain, and bromodomain (Kutateladze and SnapShot 2011). In rice, three proteins have been reported to be reader proteins: CHR729, OsSiz1, and MORF-RELATED GENE702 (MRG702) (Hu et al. 2012; Jin et al. 2015; Shindo et al. 2012). CHR729, a CHD3 chromatin-remodeling factor, binds to H3K4me2 and H3K27me3 via the chromodomains and the PHD domain, respectively (Hu et al. 2012). Mutation or downregulation of *CHR729* causes a set of morphological and growth defects. Expression analysis indicates that CHR729 mutation affects many gene expression especially those of transcription factors (Hu et al. 2012). OsSiz1 is a Siz/PIAS-type SUMO ligase, recognizing both methylated Lys4 and Arg2 of histone H3 by its PHD domain (Shindo et al. 2012). MRG702, a reader of H3K4me3 and H3K36me3, is involved in the regulation of many brassinosteroid (BR)-related and flowering genes (Jin et al. 2015).

Transposable elements (TE), which are considered to be selfish DNA, occupy large proportions of the rice genome. In eukaryote, TE can be categorized into two classes: retrotransposons and DNA transposons. In plants, retrotransposons generally contribute to variation in genome size, whereas DNA transposons preferentially insert in or near genes to generate allelic diversity, especially in the case of rice (Song and Cao 2017). The association of transposon with genes plays important roles in gene regulation, as TEs are generally silenced by epigenetic mechanisms. These mechanisms involve small RNAs, DNA methylation, and histone modifications (Cui and Cao 2014; Lisch 2009). For example, knockdown of Dicer-like 3 homolog *OsDCL3a* leads to ectopic expression of many miniature inverted-repeat transposable element (MITE)-related genes (Wei et al. 2014). These genes including gibberellin homeostasis-related genes and BR biogenesis-related genes are direct targets of 24-nt small interfering RNA (siRNA). Activation of these gene expression in *OsDCL3a* knockdown lines is caused by reduced levels of 24-nt siRNA and repressive chromatin mark H3K9me2 (Wei et al. 2014). A gain-of-function epiallele of rice *RELATED TO ABI3 AND VP16 (RAV6)*, *Epi-rav6*, was also identified (Zhang et al. 2015). It was found that a MITE inserted in the promoter of RAV6 was hypomethylated in *Epi-rav6*. The hypomethylation caused ectopic expression of RAV6 (Zhang et al. 2015). Methylome analysis provides evidence that DNA methylation changes induced by environmental or developmental cues are associated with alteration of expression of adjacent genes (Song and Cao 2017).

23.3 Implication in Development and Stress

As mentioned above, epigenetic regulation is involved in modulating many key developmental and stress-responsive gene expressions in rice, which is proved by functional analysis of histone modification enzymes. These developmental processes include plant growth, flowering time control, flower development, and seed

development. Environmental stresses such as salt, cold, drought, submergence, and disease response also involve epigenetic regulation. In general, histone acetylation is more likely to respond to various stresses, whereas histone methylation plays important roles in rice development, especially by controlling flowering time and flower development as reviewed by Shi et al. (2014).

23.3.1 Plant Growth

Histone acetylation might be involved in inhibiting plant growth. Overexpression of rice *HDAC1* leads to increased growth rate. In mature overexpression plants, the leaf blades were wider and longer, and the collar developed strongly and grew horizontally (Jang et al. 2003). Downregulation of *HDA704*, *HDT702*, and *HDA710* by RNA interference (RNAi) induces semidwarf phenotype (Hu et al. 2009). In addition, *HDA702* RNAi transgenic plants show twisted flag leaf, and *HDT702* RNAi plants display narrow leaf (Hu et al. 2009). However, insight into how histone acetylation controls plant growth has not been provided yet.

Several studies also indicate the involvement of histone methylations such as H3K4me, H3K27me, and H3K36me in controlling plant growth, by analyzing the function of enzymes related to these marks. Downregulations of both *SDG725* and *MRG702*, encoding a H3K36 methyltransferase and an H3K36me3 reader protein, respectively, cause dwarf and shortened internode phenotype (Jin et al. 2015). It has been clear that these proteins control plant growth by modulating BR biosynthesis or signaling pathway genes such as *DWARF11* (*D11*), *BRASSINOSTEROID-INSENSITIVE 1* (*BRI1*), and *BRASSINOSTEROID- UPREGULATED* (*BU1*) (Jin et al. 2015). A H3K4 demethylase, JMJ703, promotes cell division possibly by increasing cytokinin levels (Chen et al. 2013). Mutation of *JMJ703* results in upregulation of many cytokinin oxidase (CKX) genes and increased level of H3K4me3 on these genes (Chen et al. 2013). Knockout and knockdown plants of *CHR729*, encoding an H3K27me3 reader protein, also show dwarf, narrow leaf and defect in crown root formation phenotype (Hu et al. 2012; Ma et al. 2015; Wang et al. 2016; Zhao et al. 2012). These phenotypes might be caused by decreased level of gibberellin (GA) and auxin (Ma et al. 2015; Wang et al. 2016). Furthermore, T-DNA insertion mutant of *JMJ705*, encoding a H3K27 demethylase, also displays reduced plant height although detailed analysis has not been performed yet (Li et al. 2013).

23.3.2 Flowering Time Control

In rice flowering pathway, a complex regulatory network constituted by cascade connection between specific transcription factors has been built to control expression of *Heading date 3a (Hd3a)* and *RICE FLOWERING LOCUS T1 (RFT1)* (Shi

et al. 2014). Various epigenetic regulators related to histone methylation have been revealed to control flowering time by regulating these genes. JMJ701, also named Se14, is a H3K4 demethylase. Phenotypic and molecular evidences indicate that JMJ701 represses *RFT1* expression by removing H3K4 trimethylation from *RFT1* chromatin to suppress flowering under long day-length conditions (Yokoo et al. 2014). Although catalytic specificity of SDG723 on H3K4 is not defined, suppression of *SDG723* causes a late-flowering phenotype by controlling *Grain number, plant height, and heading date7 (Ghd7)* pathway. Elevated expression of *Ghd7* and decreased expression of *Early heading date1 (Ehd1)*, *Hd3a*, and *RFT1* were observed in *SDG723* suppression lines (Choi et al. 2014). Consistently, deficiency of methyl group donor, S-adenosyl-L-methionine, reduces *Ehd1*, *Hd3a*, and *RFT1* expression, which is associated with decreased levels of H3K4me3 (Li et al. 2011b). These imply the important role of H3K4me3 in the expression of flowering genes. H3K27me3 is also essential in the vegetative to reproductive transition. Firstly, genome-wide analysis indicates that a change in the H3K27me3/H3K4me3 ratio, regulated by the interaction between SDG711 and JMJ703, is an important factor for the differential expression of many genes during the transition. SDG711-mediated H3K27me3 represses several important genes involved in inflorescence meristem (IM) activity (Liu et al. 2015). Secondly, two rice E(z) genes *SDG711* and *SDG718* have been reported to accurately control flowering time in long day and short day, respectively, by regulating corresponding genes (Liu et al. 2014). Finally, a chromatin-remodeling factor VIN3-LIKE 2 (OsVIL2) repressed *LEAFY COTYLEDON 2 and FUSCA 3-LIKE 1* (*OsLFL1)* expression by interaction with EMBRYONIC FLOWER 2b (OsEMF2b), a component of polycomb repression complex2 (PRC2). Mutation of *OsVIL2* diminishes enrichment of H3K27me3 on *OsLFL1* (Yang et al. 2013). There is evidence that H3K36me3 also participates in flowering time control. The H3K36 methyltransferases including SDG725, SDG708, and SDG724 and the reader protein MRG702 promote the key flowering regulatory gene expression, and mutation of any of the four genes causes the late-flowering phenotype (Liu et al. 2016; Sui et al. 2012; Sun et al. 2012; Jin et al. 2015).

23.3.3 Flower Development

Floral organ identity is specified by various MADS-box genes. These genes show specific temporal and spatial expression patterns in different floral organ, which might involve H3K27me3. A rice *AGAMOUS* (*AG*) homolog, *OsMADS58*, is repressed by an EMF1-like protein CURVED CHIMERIC PALEA 1 (CCP1), also named DEFORMED FLORAL ORGAN1 (DFO1) (Yan et al. 2015; Zheng et al. 2015). The repression is critical for palea development. The level of H3K27me3 is reduced on *OsMADS58* chromatin in *CCP1* or *DFO1* (Yan et al. 2015; Zheng et al. 2015). In vitro and in vivo assays demonstrate that DFO1 directly interacts with the rice polycomb PRC2 proteins (OsMSI1 and OsiEZ1). These

indicate that DFO1 recruits polycomb PRC2 to *OsMADS58* chromatin to catalyze H3K27 trimethylation and mediate the repression of *OsMADS58* (Zheng et al. 2015). Rice E-class genes, *OsMADS1*, *OsMADS6*, *OsMADS34*, are also targeted by H3K27me3 and differentially expressed in loss-of-function mutant of *OsEMF2b* (Conrad et al. 2014). The mutant shows floral organ defects and indeterminacy, resembling loss-of-function mutants of E-class genes. JMJ706, a H3K9 demethylase, is required for the expression of *DEGENERATED HELL1* (*DH1*) and *OsMADS47* and affects many aspects of floral organ development (Sun and Zhou 2008).

23.3.4 Seed Development

Many epigenetic regulators, such as OsSRT1, SDG728, JMJ703, SDG725, MRG702, FERTILIZATION INDEPENDENT ENDOSPERM 1 (OsFIE1), and OsFIE2, have been reported to regulate seed development since their mutation or downregulation leads to reduced seed size (Qin et al. 2010; Cui et al. 2013; Jin et al. 2015; Huang et al. 2016; Nallamilli et al. 2013; Zhang et al. 2016a). Small grains observed in *SDG725* and *MRG702* RNAi plants may be due to BR deficiency as described above in Sect. 23.3.1 (Jin et al. 2015). OsSRT1, OsFIE1, and OsFIE2 control seed development through regulation of starch metabolism, storage protein synthesis, or amino acid metabolism-related gene expression (Huang et al. 2016; Nallamilli et al. 2013; Zhang et al. 2016a). OsSRT1 is a histone deacetylase, which is required for the repression of transposable elements by removing H3K9 acetylation from chromatin of the loci (Zhong et al. 2013). *OsFIE1* and *OsFIE2* encode ESC-like component of PRC2 and are necessary for catalyzing H3K27 methylation by PRC2. *OsFIE1* is imprinted, with the maternal allele specifically expressed in endosperm, and *OsFIE2* is expressed in a variety of tissues (Zhang et al. 2012b). H3K9 methyltransferase SDG728 and H3K4 demethylase JMJ703 also play important roles in repressing transposon activity, but how they regulate seed size is not clear (Qin et al. 2010; Cui et al. 2013).

DNA methylation also has a profound effect on gene expression in rice seed. Many genes encoding storage proteins and starch synthesis enzymes are hypomethylated (Zemach et al. 2010a). In consistent with these observations, mutation of both DNA methyltransferase MET1b and DNA demethylase ROS1a gives rise to abnormal seed development phenotype (Yamauchi et al. 2014; Ono et al. 2012). This suggests that control of DNA methylation levels in seed is important for normal development. Recent work revealed that both maternally and paternally imprinted genes were involved in control of seed development by regulating nutrient metabolism and endosperm development (Yuan et al. 2017). These genes were associated with miniature inverted-repeat transposable elements (MITEs), differentially methylated loci, or encoding siRNAs and long noncoding RNAs (lncRNAs) (Yuan et al. 2017).

23.3.5 Stress Response

Accumulated evidences reveal that epigenetic regulation is essential in stress response signaling. Firstly, expression of many epigenetic regulator genes is affected by various stresses. For examples, 7 of 8 HAT genes and 8 of 18 HDAC genes are induced or repressed by cold, drought, and salt stress (Liu et al. 2012; Fang et al. 2014; Fu et al. 2007). Most of (16 of 20) histone demethylase JmjC genes could be induced by *Xoo* infection (Hou et al. 2015). Expression of histone variant genes has also been examined to be regulated by specific stress response. H3.3 genes *HTR711* and *HTR716* are induced by brown planthopper and salt stress, respectively (Qiu et al. 2006; Wang et al. 2003). Expression of two H2A.Z genes *HTA705* and *HTA712* is repressed by salt and drought stress (Hu and Lai 2015). Secondly, genome-wide histone modification levels change in response to stress. The acetylation level of total H3, histone H3K9, H3K18, H3K27, and H4K5 is increased in response to drought treatment (Fang et al. 2014). ChIP-seq data indicates that H3K4me3 levels are increased in 3927 genes and decreased in 910 genes upon drought stress (Zong et al. 2013). However, only a small proportion of these genes are differentially expressed after drought treatment (Zong et al. 2013). H3K14 but not H3K9 or H3K27 acetylation is significantly reduced after cold treatment (Roy et al. 2014). Di- and tri-methylated H3K4, H3K9, H3K27, and H3K36 levels change with the time of *Xoo* inoculation (Hou et al. 2015). Thirdly, functional analysis indicates that several epigenetic regulators are involved in the regulation of stress-responsive genes. JMJ705 activates defense-related genes by removing H3K27me3, whereas JM704 suppresses defense negative regulator genes expression by removing H3K4me2/3 from the loci (Li et al. 2013; Hou et al. 2015). Mutation of both *JMJ704* and *JMJ705* reduces rice resistance to *Xoo* infection. HDT701 negatively regulates innate immunity by reducing H4 acetylation levels in *PRR* and other defense-related genes (Ding et al. 2012). Finally, dynamic change of histone modification on specific genes in response to stress has been analyzed. Similar to submergence-induced genes, H3K9, H3K14, and H3K27 acetylation levels are increased in different regions of *OsDREB1b* chromatin after cold treatment and recovered to initial level when return to normal temperature after cold exposure. This is consistent with the change of *OsDREB1b* expression level (Roy et al. 2014). In addition, recent paper revealed a gene, PigmS, which was involved in balancing high disease resistance and yield cost, was regulated by OsAGO4a-mediated DNA methylation (Deng et al. 2017).

23.4 Natural Variation of Rice Traits Related to Epigenetic Mechanism

Although DNA methylation is relatively stable during plant development and across generations, extensive variation of DNA methylation has been observed in different rice varieties or accessions (Li et al. 2012). This is thought to be mainly due to genome sequence variation related to repetitive sequences and transposable elements. The DNA methylation variation may contribute to phenotypic variation or development of agronomic traits during adaptation, domestication, and selection. An increasing number of QTLs of rice agronomic traits have been cloned. Among these cloned QTLs, *IDEAL PLANT ARCHITECTURE 1* (*IPA1*), also known as *WEALTHY FARMER'S PANICLE* (*WFP*), was found to be regulated by an epigenetic mechanism (Jiao et al. 2010; Miura et al. 2010). High level of *IPA1* expression in the reproductive stage is required to give rise to high grain yield by promoting panicle branching (Miura et al. 2010). In the ST-12 rice variety, higher expression level of *IPA1* and lower level of DNA methylation on *IPA1* were observed compared to Nipponbare. However, it is not clear whether upregulation of *IPA1* is caused by DNA hypomethylation, as in the candidate 2.6 kb region upstream of *IPA1*, there was no significant difference in DNA methylation (Miura et al. 2010). Recent paper reveals that *IPA1* is repressed by spread of near heterochromatin (Zhang et al. 2017b). Three tandem repeats upstream of *IPA1* could attenuate the repression by creating an open chromatin structure, which is associated with DNA hypomethylation. The finding also demonstrates that optimal level of *IPA1* expression is important for an ideal yield. Both higher and lower expression of *IPA1* will reduce yield potential (Zhang et al. 2017b). This example demonstrates that epigenetic mechanism is involved in the fine-tuning of *IPA1* expression which is of great importance for rice yield.

Epigenetic regulation is also involved in controlling rice resistance to disease. In addition to PigmS described above, *ST1*, an important component of *WRKY45*-mediated resistance, is also a target of RNA-directed DNA methylation (RdDM) (Zhang et al. 2016b). Two alleles, *WRKY45-1* and *WRKY45-2*, play opposite roles in rice resistance to *Xoo* (Tao et al. 2009). It was found that a MITE transposon element inserted in the *WRKY45-1* allele was associated with the opposite function of the two alleles. A TE-siRNA, TE-siR815, was generated from the intron of *WRKY45-1*. Activation of *WRKY45-1* could increase expression of TE-siR815, and then TE-siR815 repressed *ST1* expression by RdDM, consequently increasing rice susceptibility to *Xoo*. In contrast, overexpression of *WRKY45-2*, which lacked TE-siRNA in the intron, resulted in increased expression of *ST1* and enhanced rice resistance to *Xoo* (Zhang et al. 2016b). These results highlight the importance of epigenetic regulation of transposon-associated genes in rice defense mechanism. Considering that MITEs are mainly distributed near genes in the rice genome and display large variations in rice populations, epigenetic repression of MITE elements may be a driving force of phenotypic or trait variations in rice populations.

The role of epigenetic modification in allele-specific gene expression in hybrid rice has also been explored. For instance, siRNA-mediated DNA methylation changes are thought to lead to transcriptional differences in hybrid offspring of Nipponbare and 93-11 (Chodavarapu et al. 2012). However, most of transcriptional variations are not associated with DNA methylation changes, implicating that additional factors may be involved in the process (Chodavarapu et al. 2012). It has been reported that histone deacetylase OsHDT1 was involved in epigenetic control of parental genomic interaction for differential expression of many genes including the flowering time genes (Li et al. 2011a). Recent findings demonstrate allele-specific histone modifications (ASHMs) H3K36me3 and H3K27me3 could be inherited from the parents to the hybrid, but allele-specific gene expression (ASE) is mainly regulated by H3K36me3, rather than H3K27me3 (Guo et al. 2015). However, the role of epigenetic regulation underlining heterosis has not yet been clearly demonstrated.

23.5 Epigenetic Inheritance in Rice

As introduced earlier in this chapter, the core of epigenetics is to study how gene expression is inherited from cell to cell and even through generations. The common feature of mechanism underlying epigenetic phenomenon such as imprinting, paramutation, and epimutation is that differential DNA methylation or chromatin modification of specific loci determines expression level of the genes. DNA hypermethylation represses gene expression, while hypomethylation activates gene expression. A few studies on epigenetic inheritance in rice have been reported. *OsFIE1* is a rice imprinted gene that is expressed only in endosperm, where the maternal copy is active but the paternal copy is not (Luo et al. 2009). An epiallele of *OsFIE1* is found to be hypomethylated in the 5′ region of *OsFIE1*, which leads to ectopic expression of the gene (Zhang et al. 2012b). The allele causes dwarf and floral organ defects and is inherited in a dominant fashion (Zhang et al. 2012b). Another epimutation at *Os03g02470* locus was identified, which is induced by callus culture and could be inherited stably through generations (Chen et al. 2015). Detailed analysis indicates that *Os03g02470* is not expressed in normal plant, but after callus culture, transcripts of the gene could be detected in regenerated plant and in subsequent generations. As expected, DNA methylation is lost in the promoter and 5′ region of the gene in regenerated plants. However, overexpression of *JMJ703* and mutation of *CHR729*, which leads to decreased H3K4me3 levels, suppress the gene expression. Increased H3K27me3 levels by overexpression of SDG711 are also involved in the repression of the gene (Chen et al. 2015). These demonstrate the synergistic effects of histone modification and DNA methylation on the control of epiallele. Several studies have also revealed that various stress-induced variations in DNA methylation at specific loci could be inherited (Feng et al. 2012; Kou et al. 2011; Ou et al. 2009, 2012; Zheng et al. 2013). Alteration of DNA methylation does not show a causal relationship with

alteration of gene expression (Ou et al. 2009). However, the rice plants growing under a specific stress condition for several generations show enhanced tolerance to the stress compared with the plants growing under normal condition (Feng et al. 2012; Kou et al. 2011; Ou et al. 2009, 2012; Zheng et al. 2013). In addition, two kinds of rice varieties with distinct salt stress sensitivity show different altered DNA methylation patterns after growing under stress condition (Feng et al. 2012). Generally, DNA methylation level is increased in salt-tolerance genotypes and decreased in salt-sensitive genotypes, implying the importance of DNA methylation in the adaption of salt stress. It would be intriguing to reveal how inheritance of DNA methylation pattern is related to the adaptive traits.

23.5.1 Perspectives

Accumulating epigenomic data provides useful information for understanding features and functions of the rice epigenome. Large amounts of chromatin modification regulators including writers, erasers, and readers have been characterized. It is still not very clear how these regulators are involved in the establishment, maintenance, recognition, and erasure of rice epigenomes and how epigenomic marks are maintained or inherited after cell division. Future research in the field of epigenetics will focus on the mechanism how epigenomic marks established in specific cell types or in responding to specific environmental cues can be memorized during cell divisions and inherited to next generations. Moreover, since several natural variations of traits have been proved to be caused by epigenetic mutations, how epigenetic mechanism of gene expression can be applied to improve agronomic traits is of great importance.

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Chapter 24
Genomic Selection in Rice Breeding

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Abstract Genomic selection (GS) is a new breeding method that makes use of genome-wide DNA marker data to improve the efficiency of breeding for quantitative traits. In GS, individuals with superior breeding values are identified and selected based on prediction models built by correlating phenotype and genotype in a breeding population of interest. The potential of GS to improve rice breeding efficiency has recently been evidenced by a number of empirical and simulation studies; however efforts to implement GS in rice breeding are still limited, particularly as compared to other major grain crops such as maize and wheat. In this chapter, we discuss a variety of GS modeling methods, practical considerations for implementing GS in rice breeding programs, and the rapid evolution of GS technology. We conclude with a discussion of what this means for GS technology in the future.

Keywords Whole-genome selection · Genomic prediction · Breeding values · Prediction models · Statistical models · Implementation in rice breeding · Omics-aided breeding

24.1 Overview of Genomic Selection

Since Meuwissen et al. (2001) introduced their seminal paper on genomic selection (GS) at the beginning of the twenty-first century, GS has attracted great interest as a means of accelerating the rate of genetic improvement in both livestock (Hayes et al. 2009b; VanRaden et al. 2009) and crop breeding (Bernardo and Yu 2007; Heffner et al. 2009; Jannink et al. 2010; Lorenz et al. 2011, Desta and Ortiz 2014).

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In their simulation study, Meuwissen and colleagues found that the breeding values of individuals could be accurately predicted based on ~1010 DNA polymorphisms located at equal intervals throughout the genome. The result suggested a breeding application in which individuals with superior breeding values could be accurately selected using prediction models based on genome-wide DNA marker data. This suggested a potentially large time advantage because, for many breeding programs, there is a delay between when genotyping can be performed on a breeding population and selections made and when phenotyping can be performed. For example, this time advantage was first seized by dairy cattle breeders, for whom this delay was particularly long. Prior to genomic prediction technology, dairy cattle breeders had to wait years for daughters to mature and produce milk before they could estimate the breeding value of the sire (Meuwissen et al. 2001). By replacing progeny testing with genomic prediction, genetic gain could be expected to roughly double. GS has since been rapidly applied to dairy cattle breeding (Hayes et al., 2009b; VanRaden et al. 2009) and has been credited with rapid increase of genetic gains per year in US Holstein dairy cattle breeding (García-Ruiz et al. 2016). The successful application of GS to livestock breeding had important implications for GS in crops, and as a result, it has also been utilized or evaluated in a large variety of crop species including, but not limited to, maize, wheat, rye, oats, soybeans, trees, and of interest to us in this chapter – rice (Xu et al. 2014; Onogi et al. 2015; Grenier et al. 2015; Spindel et al. 2015, 2016; Wang et al. 2017).

In GS, a set of individuals that resembles the breeding population is selected to form the training set or training population. These individuals are genotyped with the genome-wide markers and also phenotyped for one or more traits of interest. These data are then used to build a prediction model that essentially functions to correlate genotype with phenotype – or, in other words, fed a set of genotype data the model outputs estimates of breeding value known as genome-estimated breeding values or GEBVs. This allows the breeding value of individual lines/plants to be predicted based on genotype alone. Lines with highest GEBVs can then be selected for advancement within a GS breeding program, or alternatively, lines with the lowest GEBVs can be removed.

GS differs from conventional phenotypic selection in that, in conventional phenotypic selection, the only way to obtain an estimate of line breeding values is to repeatedly measure the performance of an individual or family in the field. Such field trials, however, require a great deal of time and resources both to grow and manage the plants and to collect accurate phenotype data. Moreover, because field trials are subject to heterogeneous environmental conditions, it is necessary to repeat the tests over many years at the target locations. This greatly prolongs variety development and increases costs. In GS programs, in theory, repeated field trials are not necessary because breeding values are instead quickly estimated using genome-wide markers. In addition, cultivation of lines/plants does not necessarily have to take place in the target environments. For example, selection can be performed multiple times a year by using off-season nurseries or artificial environments (Ohnishi et al. 2011; Tanaka et al. 2016). This is known as “remote breeding,” i.e., the development of new breeding materials for target locations at remote

locations (Manickavelu et al. 2017). Furthermore, in some cases, GS is particularly useful for traits that are difficult or expensive to measure, e.g., sensory properties of cooked rice (Lau et al. 2015), because the measurement is only necessary on the subset of lines/plants used to build the prediction model.

The idea of selecting lines/plants using DNA markers without measuring phenotypes of target traits is not unique to GS. Marker-assisted selection (MAS) is another method that has been used to select traits based on DNA markers. However, traditional MAS is only useful when selecting on one or a very few QTL or gene loci and cannot be used to select for quantitative traits controlled by many loci of small effect, such as yield (Bernardo 2008). This is because traditional MAS is performed using only a small number of markers, chosen because they are linked to known large-effect QTL or other regions of interest. By contrast, GS is performed using a large number of genome-wide markers, without any distinction between those that are linked to QTL or genes of interest and those that are not. This means that even very minor effect loci can and will be captured by GS models, as long as there are genome-wide markers in linkage disequilibrium (LD) with the respective QTL present in the model. The superiority of GS over traditional MAS for the improvement of complex traits has been supported by many simulation and empirical experiments (Bernardo and Yu 2007; Heffner et al. 2011; Wang et al. 2014; Arruda et al. 2016; Zhang et al. 2016b).

The potential of GS in rice breeding, in particular, has been recently evidenced by a number of empirical and simulation studies (Xu et al. 2014; Onogi et al. 2015; Grenier et al. 2015; Spindel et al. 2015, 2016; Yabe et al. 2016; Marulanda et al. 2016; Wang et al. 2017). Efforts to implement GS in rice breeding, however, are still limited, especially when compared to other major crops such as maize or wheat. In part this may be due to the history of breeding and genetics research in rice. In the past, many studies were devoted to the mapping and cloning of quantitative trait genes, and GS is based on a fundamentally different philosophy. Logistical and infrastructure challenges also exist – many rice breeding programs are small and low-resource, which is a problem given the current expenses associated with genotyping and bioinformatics analyses necessary to implement GS. In the future, new public-public and public-private partnerships will be necessary to overcome these challenges.

In this chapter, we introduce the modeling methods used in GS and discuss some of the practical aspects involved in implementing GS in rice breeding. Finally, we will discuss how GS technology is changing quickly and what this might mean for GS technology in the future.

24.2 Modeling Methods for Genomic Selection

In genomic selection, a model predicting the breeding values of a target trait based on genome-wide marker genotypes is used for the selection. Now, let $\mathbf{x}_i$ and y_i represent the genome-wide marker genotype and breeding values of individual i, respectively. Then, a prediction model $f(\mathbf{x}_i; \boldsymbol{\theta})$ can be described as

$$y_i = f(\mathbf{x}_i; \boldsymbol{\theta}) + e_i$$

where $\boldsymbol{\theta}$ is a vector of parameters included in the model and e_i is a residual. Single nucleotide polymorphisms (SNPs) are generally used as genome-wide markers. Three genotypes of biallelic SNP (e.g., TT, AT, AA) are scored as $=1$, 0, and -1 (or 0,1,2). For example, when 50,000 SNPs are used for the prediction, $\boldsymbol{x}_i$ is expressed as a vector of 50,000 dimensions, which has -1, 0, or 1 (or 0,1,2) as each element. The model is built with "training data" which consist of the genome-wide marker genotypes and breeding (or phenotypic) values of cultivars and lines/plants belonging to breeding populations. More specifically, in the model building, the estimated values of model parameters, $\widehat{\boldsymbol{\theta}}$, are obtained using the training data. Once the estimated values, $\widehat{\boldsymbol{\theta}}$, are obtained, the breeding values of the individual s can be predicted as $f\left(\mathbf{x}_s; \widehat{\boldsymbol{\theta}}\right)$ based on its genome-wide marker genotype, $\mathbf{x}_s$. In the first paper on GS, Meuwissen et al. (2001) proposed a linear regression model that regressed the breeding value y_i to the genome-wide marker genotype, $\mathbf{x}_i$:

$$f(\mathbf{x}_i; \boldsymbol{\theta}) = \beta_0 + \sum_{j=1}^{M} x_{ij}\beta_j$$

where β_0 is the intercept and β_j $(j = 1, 2, \ldots, M)$ is the genetic effect of the allele of SNP marker j and x_{ij} is the j-th element of $\mathbf{x}_i$. By applying this linear regression model to the training data and obtaining the estimated parameter values $\widehat{\boldsymbol{\theta}} = \left(\widehat{\beta}_0, \widehat{\beta}_1, \ldots, \widehat{\beta}_M\right)'$, the breeding value of s-th individual can be predicted as

$$\tilde{y}_s = \widehat{\beta}_0 + \sum_{j=1}^{M} x_{sj}\widehat{\beta}_j$$

where $\tilde{y}_s$ is the predicted breeding value of individual s.

In GS, the number of genome-wide markers, M, usually exceeds the number of samples (e.g., cultivars and lines/plants in breeding populations), N, in the training data (González-Recio et al. 2014). In crops, N generally ranges from several hundred to several thousand individuals, while M has rapidly increased in recent years and usually ranges from tens of thousands to hundreds of thousands. Thus, data for training the GS model is characterized by what is known as the "*large p small n*" problem, in which the number of markers (p) far exceeds the number of samples (n). Furthermore, since markers located close to each other are generally in

strong linkage disequilibrium (LD), the multicollinearity among explanatory variables is quite high. Genomic prediction models must thus address both of these issues.

A large number of methods have been proposed for constructing GS prediction models (González-Recio et al. 2014; de los Campos et al. 2013; Kärkkäinen and Sillanpää 2012; Garrick et al. 2014; Jacquin et al. 2016; Xavier et al. 2016). The most important requirement for a GS model is high accuracy; thus any method can be used as long as an accurate prediction model is obtained. The most commonly used method of building a GS model is genomic best linear unbiased predictor (G-BLUP).

Best linear unbiased predictor (BLUP) based on pedigree records has a long history in animal and plant breeding programs. G-BLUP works by replacing the pedigree-based numerator relationship matrix in traditional BLUP with a genomic relationship matrix calculated from genome-wide marker genotypes (Habier et al. 2007). In order to address the "*large p small n*" problem, a regression model using a regularization method is also frequently used, e.g., ridge regression, LASSO, or elastic net. Among these, ridge regression is synonymous with G-BLUP (Jacquin et al. 2016; Hayes et al. 2009a; Meuwissen et al. 2016) and is frequently referred to as RR-BLUP (ridge regression BLUP or random regression BLUP).

Bayesian regression models (de los Campos et al. 2013; Kärkkäinen and Sillanpää 2012) are also used as the Bayesian counterpart of frequentist regularization methods. Different Bayesian models assume different prior distributions for individual marker effects (β_j in the linear regression model described above) and are often called by names that include capital letters of the alphabet, such as Bayes A and Bayes B (Gianola 2013), and are thus collectively often referred to as the "Bayesian alphabet." Representative examples include Bayesian Ridge Regression, Bayesian LASSO (Park and Casella 2008), Bayes A, Bayes B (Meuwissen et al. 2001), Bayes Cπ, and Bayes R (Habier et al. 2011). Bayesian Ridge Regression and Bayesian LASSO are Bayesian counterparts of ridge regression and LASSO, respectively (de los Campos et al. 2013; Jacquin et al. 2016). Bayes A, B, Cπ, and R differ only in the assumed prior distributions of marker effects. Bayes A assumes a prior distribution of marker effects that follows a scaled t distribution; Bayes B, a zero or a scaled-t distribution; Bayes Cπ, a zero or normal distribution; and Bayes R, a mixed distribution of three or more normal distributions.

All of the models described above assume that marker effects are linear and additive. There are also nonlinear GS models that do not make these assumptions. One such method is an extension of G-BLUP. Dominance effects, or dominant deviations, are introduced into linear mixed models similar to G-BLUP by calculating a dominant genomic relationship matrix (Vitezica et al. 2013). As suggested by Henderson (1985), epistatic effects can also be introduced into linear mixed models by calculating epistatic genomic relationship matrices as the Hadamard product of genomic relationship matrices (Jiang and Reif 2015).

Alternatively, nonlinear kernel regression may be used, such as reproducing kernel Hilbert spaces (RKHS) regression (Gianola and van Kaam 2008). As shown by Jiang and Reif (2015), RKHS with a radial basis function (RBF) kernel can

capture epistatic effects estimated based on epistatic genomic relationship matrices. Although an RBF kernel is often used, other kernels specific to GS have also been proposed (Morota et al. 2013). BLUP methods based on pedigree information (traditional BLUP) and G-BLUP can both be considered types of kernel regression, in which the relationship matrix is a linear kernel (Jacquin et al. 2016; Xavier et al. 2016; Gianola et al. 2006; Endelman 2011). Kernel partial least squares (PLS) regression and kernel-based neural network have also been proposed as GS methods (Iwata et al. 2015; Gonzàlez-Camacho et al. 2012, 2016) and in the prediction of human disease level (Yang et al. 2016).

Various regression methods in the machine-learning field have been also used in GS (González-Recio et al. 2014). Support vector machine (Vapnik 1995) and random forest (Breiman 2001) are two commonly used machine-learning methods for regression and classification (Iwata and Jannink 2011; Spindel et al. 2015; Onogi et al. 2015; Heslot et al. 2012). Support vector machine regression is a kernel regression method and is similar to RKHS in that it is good at modeling nonlinear genetic effects. A special feature of support vector machine regression is its "ε-insensitive loss function," which is intended to minimize the regression sum of absolute errors with ignoring small errors. Random forest, which is a tree-based regression method, has also frequently been found to outperform linear regression methods (Iwata and Jannink 2011; Onogi et al. 2015; Spindel et al. 2015).

The Bayesian counterpart of tree-based regression methods has also been found to produce accurate genomic predictions (Waldmann 2016). Other machine-learning methods, including boosting, bagging, and neural networks, have also been used to build GS models (González-Recio et al. 2010; Gianola et al. 2011, 2014).

In GS, prediction is generally treated as a regression problem, i.e., the problem predicting the continuous feature of a data point. A different approach, in which the prediction is treated as a classification problem, i.e., the problem predicting the class to which a data point belongs, has also been used in GS (Ornella et al. 2014; González-Camacho et al. 2016). In this approach, lines/plants are categorized into several classes (e.g., three classes of upper, middle, and lower phenotypic values) so that machine-learning methods for classification can be applied. Because the ranking of lines/plants in a target trait, especially for the higher rank, is important information for selection, machine-learning methods for predicting ranking itself have also been proposed for use in GS (Blondel et al. 2015). Machine-learning methods are evolving very quickly and have great potential for improving the accuracy of prediction in GS.

Generally, in a breeding program, more than one trait is targeted for selection, and often, these traits are correlated, e.g., yield and yield component traits are all correlated, either positively or negatively (Hori et al. 2016; Wang et al. 2017). Single-trait phenotypes measured in multiple environments can also be thought of as multiple, correlated traits; as even when genotype-by-environment (GxE) interaction is significant, phenotypes are expected to be correlated to some extent (Wang et al. 2017). Phenotypes that are measured at different growth stages can also be thought of as multiple, correlated traits (Sun et al. 2017b). One way to both enable

selection on multiple traits and leverage information sharing among correlated traits is use of multivariate (MV) genomic prediction models. MV genomic prediction models that incorporate correlated traits can improve prediction accuracy because each trait borrows information required for prediction from the other traits in the model. A model predicting phenotypes of multiple traits can thus make use of the correlation relationships among traits, where a single-trait genomic prediction model cannot.

Many of the methods mentioned above have multivariate versions that can be used for genomic prediction. Multivariate G-BLUP, which is the extension of multivariate BLUP (Henderson and Quaas 1976), is frequently used for genomic prediction. Multivariate G-BLUP with additive and dominance effects has been applied to the genomic prediction of rice hybrid performance in yield and yield-related traits (Wang et al. 2017). Multivariate versions of Bayes A, Bayes B, and Bayes Cπ have also been proposed (Calus and Veerkamp 2011; Jia and Jannink 2012; Hayashi and Iwata 2013). Kernel PLS regression, which can be applied to high-dimensional traits, was used to predict of rice grain shape (Iwata et al. 2015). As demonstrated by Scutari et al. (2014), Bayesian networks, which can also be used for multi-trait genomic prediction, enable the graphical interpretation of complex relationships among traits and markers.

Multi-trait genomic prediction also enables the prediction of a target trait using the phenotypes of secondary traits as auxiliary variables (Rutkoski et al. 2016; Wang et al. 2017; Sun et al. 2017b). This feature allows us to predict traits that are difficult or expensive to measure based on traits that are easy or inexpensive to measure. For example, yield can be predicted based on the observed values of yield-related secondary traits, such as canopy temperature and vegetation indices, that can be measured in a high-throughput manner at early growth stages (Rutkoski et al. 2016; Sun et al. 2017b). Multi-trait genomic prediction can also be used as a method for imputing missing data in phenotypic records (Dahl et al. 2016; Hori et al. 2016).

When implementing GS in a breeding program, it is important to choose the appropriate statistical method among all of these options. In rice, there have been several empirical and simulation studies aimed at comparing the accuracy of different GS methods (Onogi et al. 2015; Grenier et al. 2015; Spindel et al. 2015, 2016). The results suggest no single method is best for all traits, i.e., there is "no free lunch." This result is explained by the fact that genomic prediction accuracy depends on numerous factors that vary between traits, including but not necessarily limited to the number of genes controlling the trait of interest, the distribution of trait allele effects, the presence or absence of epistasis, and the trait heritability (Iwata and Jannink 2011; Onogi et al. 2015). Onogi et al. (2015), conducted a simulation analysis based on real marker data to investigate the influence of these factors as well as the training population size. In the presence of epistasis, the accuracy of RKHS and random forest was high, while G-BLUP and LASSO were found to be most accurate in the absence of epistasis. Bayesian LASSO and its related approaches showed stable performance under both conditions. Because prior knowledge about a target trait is usually not sufficient to determine best modeling method for a particular trait, it is necessary to evaluate the accuracy of

GS empirically via cross-validation of the training population or via validation using target breeding populations before implementing GS in an applied breeding program (Xu et al. 2014; Yamamoto et al. 2017; Minamikawa et al. 2017). The latter is a more reliable way of evaluating accuracy than the former, because the pattern of linkage disequilibrium may differ between training populations and breeding populations.

This having been said, it is important to note that in the vast majority of empirical studies of GS, the difference in the accuracies of various genomic prediction modeling methods has generally been found to be quite small (Heslot et al. 2012; Onogi et al. 2015). However, it is also important to note that prediction accuracy in the initial breeding population (i.e., the first selection cycle) will not be the same as the prediction accuracies in future generations obtained through repeated cycles of crossing and selection. Crossing and selection changes the relationship between genome-wide marker genotypes and phenotypes by breaking marker-gene linkages, and thus the accuracy of the predictive model is expected to decline over time (Jannink 2010). The extent of this reduction can vary greatly between methods (Habier et al. 2007). Prediction in GS utilizes not only LD between QTL and markers but also the genetic relationships between plants in the population, and the dependency of models on LD and the family relationship differ among methods (Habier et al. 2007; Onogi et al. 2015). For example, Bayes B depends more on LD, while G-BLUP depends more on the genetic relationship (Habier et al. 2007). The latter type of method is expected to be less accurate over time than the former, as the genetic relationship decays with each generation of recombination. Simulation analysis and a system for performing the analysis will be useful and necessary to evaluate the robustness of models over generations and to clarify the required frequency of model updating (Jannink 2010; Yabe et al. 2017).

The current technologies for sequencing allow us to collect whole-genome sequences of a large number of genotypes. In rice, the project of sequencing 3000 rice accessions has been taken places (The 3,000 rice genomes project 2014), and the whole-genome sequences of a large number of rice varieties/lines are already available in public databases (Sun et al. 2017a). The sequencing technologies are still evolving toward higher efficiency and lower price and will accelerate this trend. The whole-genome sequences can be used for genomic prediction and may improve the accuracy of the prediction because the whole-genome sequences are expected to include the causal mutations that underlie QTL or at least to increase the degree of linkage disequilibrium between SNPs and QTL (Meuwissen and Goddard 2010). Based on the genotypes of high-density genome-wide markers, the whole-genome sequences of lines/plants can be imputed by using whole-genome sequences available in databases as a reference panel (Jannink et al. 2009; Iwata and Jannink 2010).

Genomic selection based on whole-genome sequences, however, may have drawback from increasing the number of genome-wide markers. A large number of markers not only affect the computational efficiency for building a genomic prediction model but can also lead to a poor performance of GS, mainly due to possible overfitting and/or uninformative markers (He et al. 2016). Selection or

weighting of informative markers, i.e., feature selection or weighting in a machine-learning sense, is expected to improve the efficiency of model building and accuracy of GS. Several approaches have been proposed to selecting or weighting informative markers. Genome-wide association study (GWAS) is one of the ways to selecting markers (Spindel et al. 2016; Veroneze et al. 2016; Veerkamp et al. 2016; Bian and Holland 2017). Spindel et al. (2016) proposed to select markers with GWAS with a training population ("de novo" GWAS) and to use markers as fixed effects in a BLUP model. G-BLUP is also used for evaluating the importance of markers. Zhang et al. (2014) proposed a G-BLUP method that can incorporate prior knowledge obtained from GWAS results in public databases. As suggested by Wang et al. (2012), marker effects can be estimated from predicted breeding values and can be used as criterion for selecting markers or as weights for weighting markers (Su et al. 2014; Zhang et al. 2016a). Haplotype blocks (Cuyabano et al. 2014, 2015), which were build based on the LD between adjacent markers, reduce the number of variables without the loss of information and can improve the reliability of genomic prediction compared to an individual SNP model. Mutual information, which is a measure of dependency between two variables, is useful to select markers that have maximum relevance with phenotypes and markers that have minimum redundancy between markers (He et al. 2016). Biological information, such as genome annotation (Sveinbjornsson et al. 2016; MacLead et al. 2016) and gene ontology (Edwards et al. 2016), are also useful to selecting or weighting informative markers. Speed and Balding (2014) extended a BLUP model to include multiple random effects that correspond to the effects of classes of SNPs, e.g., classes specified SNP functional annotation.

Although some studies suggest GS accuracy can be improved by using whole-genome sequences (Meuwissen and Goddard 2010; Iheshiulor et al. 2016), most studies do not find a significant increase of the accuracy (Pérez-Enciso et al. 2015; van Binsbergen et al. 2015; Calus et al. 2016; Heidaritabar et al. 2016; Veerkamp et al. 2016; Ni et al. 2017). To leverage the information contained in the whole-genome sequences in GS, further studies will be necessary.

24.3 Genomic Selection of Rice in Practice

When it comes to implementing GS into a new or existing rice breeding program, the first questions are usually not statistical but logistical. What should compose the training population? How much genotyping is necessary, and what platform should be used? How often should the model be updated? When should selections be made, and how should GS be used if working with a primarily inbred crop such as rice? This section addresses the questions breeders must answer when considering implementing GS into their programs.

24.3.1 Training Population Design

The purpose of the training population in a GS breeding program is to accurately sample the full breadth of genetic diversity found in the breeding population so that the relationship between genotype and phenotype can be accurately correlated and modeled. Thus, regardless of model type, the selection of a proper training population is critical to the success of any GS program. When designing a training population, several important factors need to be considered, including (1) the training population size, (2) the relationship between the training population and the breeding population, (3) genetic structure within the training population, and (4) how and when the genomic model will be updated over the course of the breeding program, i.e., what, if anything, will constitute the *retraining* populations during future cycles of selection?

How closely the training population resembles the final breeding (a.k.a. validation) population has one of the largest effects on prediction accuracy of any component in genomic breeding program design (Desta and Ortiz 2014; Bassi et al. 2016; Jannink et al. 2010). There is consensus in the literature that accuracies are highest when individuals in a training population are closely related to those in the testing population and that accuracies drop rapidly as genetic distance between the two groups increases. For example, when full-sibs compose the training and testing populations, accuracies have been shown to be very high in a wide variety of crops and environmental conditions (Guo et al. 2012; Lian et al. 2014; Lorenzana and Bernardo 2009; Massman et al. 2013a, b; Peiffer et al. 2014; Riedelsheimer et al. 2013; Zhao et al. 2012; Albrecht et al. 2011; Zhang et al. 2015; Ornella et al. 2012; Schulz-Streeck et al. 2012; Charmet et al. 2014), but Lehermeier et al. (2014) found that, depending on the trait, 375 to more than 675 half-sib lines were needed to equal the predictive power of just 50 full-sibs. Within multi-parent families, relatedness is just as important – when genetically distinct populations compose the training and testing populations, prediction accuracies have repeatedly been found to be poor (Windhausen et al. 2012; Asoro et al. 2011; Lorenz et al. 2012; Schopp et al. 2017; Grenier et al. 2015). Thus, when breeders are designing training populations for GS programs, they should seek training populations that resemble the breeding population as closely as possible.

The necessary size of a training population is linked to the question of relatedness, as well as other factors including trait heritability and population structure. In general, the larger the training population, the better the genomic prediction accuracies, up to a certain training population size, at which point accuracies have generally been observed to plateau (Heffner et al. 2011; Lorenz et al. 2012). The plateau represents the point at which the full diversity of the breeding population has been assayed. Thus, the ideal training population size is smaller for a closely related population and larger for a more distantly related multi-parent population. Full-sib populations, for example, have been shown to need a training population of as few as 50 individuals; however in simulations of more distantly related populations, 300 individuals seem to be necessary, at minimum (Schulz-

Streeck et al. 2012; Riedelsheimer et al. 2013; Bentley et al. 2014; Bassi et al. 2016). For the most distantly related populations, a training population as large as 1000 individuals may be necessary (Bassi et al. 2016).

Genetic structure can complicate the design of a genomic prediction experiment and selection of the training population. When multiple, distinct genetic subgroups exist within the training and testing populations, the resulting predictions will be based, in large part, on difference between the phenotypic means between subpopulations. This inflates prediction accuracies while obscuring the effect of Mendelian segregation on breeding values, i.e., the effects that are intended to be estimated by the GS model (Windhausen et al. 2012; Guo et al. 2014; Lehermeier et al. 2015). Guo et al. (2014), for example, found that up to 62% of genomic prediction accuracy in a diversity panel of rice was the result of uncorrected population structure, alone.

Thus, if not working within a structured cross such as a biparental population, care must be taken to control for population structure. Several strategies exist for addressing this problem. The first is to simply perform selection within a given subpopulation. Since most breeding programs already work within a subpopulation, this solution is often a practical one, and in these cases, population structure should be minimized. However, there are times when working within a subpopulation is undesirable. Combining subpopulations increases diversity, and sample size and subpopulations are frequently crossed to each other in hybrid breeding programs. Thus, another commonly used solution is to include principle components estimated from the genotype matrix into a G-BLUP model for genomic prediction. This represents a mean correction for population structure; however it is frequently inadequate, especially in species like rice that are subject to very deep subpopulation structure, because it does not allow marker effects to differ between subpopulations (Lehermeier et al. 2015; Yang et al. 2010; de los Campos and Sorensen 2014). As an alternative, several new models have been proposed that allow marker effects to vary between subpopulations and borrow correlated information among relatives. However, there is not yet evidence that such models can adequately control for population structure or improve prediction accuracy over simple stratified models (i.e., performing GS within subpopulations) in rice diversity panels (Schulz-Streeck et al. 2012; Lehermeier et al. 2015).

A useful rule of thumb breeders should keep in mind when designing training populations that the more correlated, quality data used to compose a training population, the better the overall accuracy of genomic predictions. This correlated data can take multiple forms including using historical phenotype data or phenotype data from correlated locations or seasons. In a multi-parent population of irrigated rice breeding lines from the International Rice Research Institute, prediction accuracies were found to improve when training data incorporated phenotypes from both wet and dry seasons, as well as across multiple research sites at related latitudes (Spindel et al. 2015, 2016). Similar results have been found in a wide variety of other crops and studies; however, accuracies have also been found to decrease if historical or multisite data are not positively correlated with the breeding population under selection, so care must always be taken when using this

approach (Asoro et al. 2011; Burgueño et al. 2012; Ornella et al. 2012; Kleinknecht et al. 2013; Bentley et al. 2014; Spindel et al. 2015, 2016; Zhang et al. 2015; Rutkoski et al. 2015; Lopez-Cruz et al. 2015).

Over the course of a GS breeding program, model retraining is necessary because the introduction of new genetic variation, recombination (i.e., crossing), selection, and genetic drift all degrade the original correlations used between genotype and phenotype in the initial training population used to build the GS model. This means that in the absence of model retraining, prediction accuracies will tend to decrease, sometime dramatically, over time (Sallam et al. 2015). Furthermore, because retraining accumulates additional, correlated, high-quality data into the model, even in the absence of the above issues, model retraining is a powerful means of improving prediction accuracies. A majority of empirical and simulation studies suggest that, ideally, some form of model retraining be performed during each round of selection; however, the exact extent to which this is necessary is highly case specific and will depend on the breeding program design (Rutkoski et al. 2015; Auinger et al. 2016). A common strategy that has been performed in several empirical studies of GS, to date, has been to phenotype a subset of the breeding population each season and uses this data to retrain the model (Auinger et al. 2016; Battenfield et al. 2016).

24.3.2 Incorporating GS into Existing Rice Breeding Programs

The ultimate goal of a plant breeding program is to make genetic gain over time for one or more traits of interest. The objective of a genomics-assisted breeding program should thus be to improve gain from selection per unit time over alternative breeding methods, generally phenotypic selection (PS) or marker-assisted selection (MAS) (i.e., selection using genotype data from only a very few markers). There are multiple ways in which GS programs can meet this objective. For example, if the GS model produces better predictions of breeding value than PS in the same amount of time, genetic gain per unit time will be greater in the GS program. Alternatively, if less time or fewer resources are required to make selections using GS relative to PS, and accuracies are maintained, then gain per unit time will also be higher for GS than for PS. The best GS programs seek to optimize both avenues, i.e., decrease necessary intergenerational time while also increasing accuracy (Bassi et al. 2016; Spindel et al. 2015; Heffner et al. 2010; Heslot et al. 2015).

Rice, as a primarily inbred crop, has generally been bred using complex pedigree schemas. GS can be incorporated along with phenotypic selection into such pedigree schemes in several ways. One design is to use GEBVs early in the pedigree line fixation process to eliminate the worst performers, thus saving time and resources that would otherwise be needed to carry lines to complete fixation. GS can also be

used later during line fixation or after lines have been fixed, to make positive selections of individuals to carry forward into yield trials. Finally, it would be natural to use GEBVs in the final round of selection to choose parents for future rounds of crossing (Fig. 24.1). Combining selection based on GEBV with rapid generation advance could further increase gain from selection per unit time (Spindel et al. 2015).

At the time of this chapter writing, there have not yet been any empirical tests of GS breeding outcomes in rice. However, many GS trials have been performed in inbreeding populations of wheat and other small grains. The majority of these pilot breeding programs have been variations on recurrent selection schemes. In general, narrow biparental crosses are made, and individuals are then selected based on GEBV, crossed, and used directly for future rounds of selection. GS can be used in such schemas as early as the F_2 population, and when selection intensity is high, intergenerational time can be drastically reduced (Bassi et al. 2016; Heslot et al. 2015). When compared to phenotypic selection, these schemes have generally been shown to improve genetic gain per unit time. They also have the advantage that they can reduce generation time even for tropical crops that do not require winter nurseries, as a major way the GS improves gain per unit time in temperate crops by enabling selection in off-season nurseries (Auinger et al. 2016; Battenfield et al. 2016; Asoro et al. 2013; Massman et al. 2013a,b; Beyene et al. 2015). Unfortunately, in rice, this type of recurrent selection program is likely to be very difficult to implement without a dominant male sterility system, which is not yet publicly available to rice breeders.

A third possible way to incorporate benefits of GS into rice breeding programs is to focus on applying GS early during preliminary yield trials when seed is limited, which prevents breeders from increasing accuracy by planting additional phenotype replicates or on traits that are very time-consuming, expensive, or laborious to phenotype. In the former case, early breeding accuracies can be boosted beyond what would normally be possible in a seed or time-limited scenario, as the only theoretical limitation to GS accuracy is the heritability of the trait. While, in the latter case, the use of GS could save time and money by forgoing much of the expensive and lengthy phenotyping (Battenfield et al. 2016; Michel et al. 2017).

GS is ultimately a conservative method of breeding, as only variation that is proven beneficial in the training population will be selected. However, it is possible to modify GS breeding programs to introduce exotic genetic diversity into established breeding programs and thus widen the genetic base of elite breeding germplasm. For example, a program could begin with the cross of an exotic parent to an adapted parent, after which GS + GWAS models could be used to rapidly introgress favorable alleles from the exotic germplasm into the adapted background, a process that can normally be very time-consuming with no guarantee of success, especially for quantitative traits (Spindel et al. 2016; Bernardo 2016). Simulations performed by Bernardo (2016) found generally positive gain from selection when GS was used to introduce exotic alleles into adapted germplasm via recurrent backcrossing. Furthermore, the number of favorable alleles from the donor parent was found to increase over time as long as an F_2 population was used

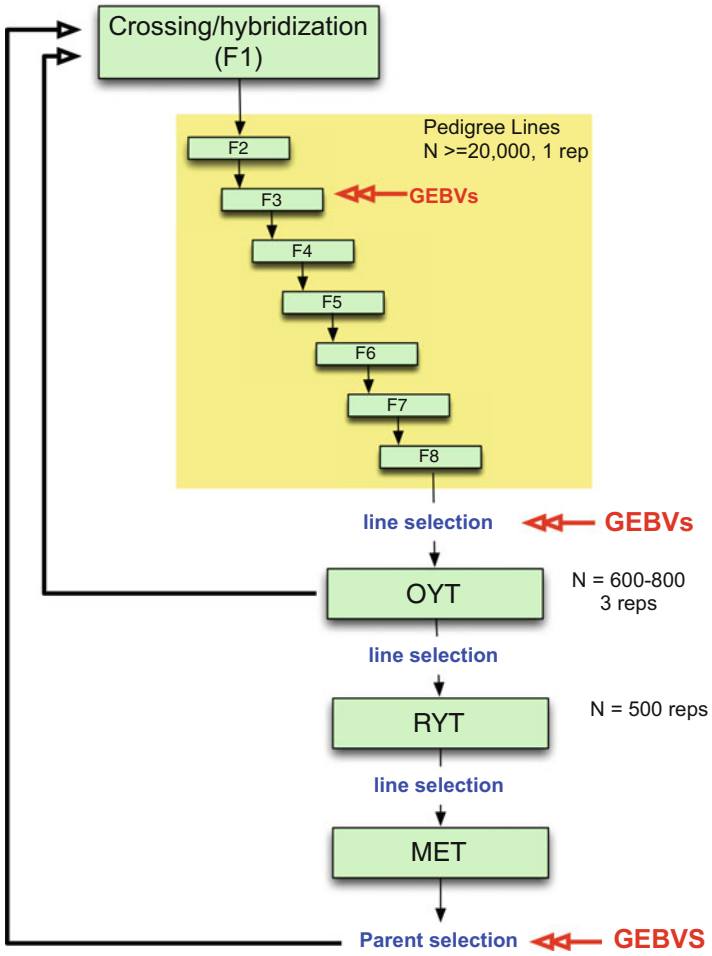

Fig. 24.1 Example of irrigated rice breeding pipeline that incorporates genomic selection. Parents are selected and crossed to create an F1 population. ~20,000 F1 lines are fixed over seven to eight generations with selection of families for heritable traits with ~25% of the pedigree lines eventually selected for entry into the observational yield trial (OYT). GEBVs can be used at two or more generations during fixation as resources permit to perform selection. Here we propose using GEBVs at the F3 and F6 generations. GEBVs are also used to select the fixed lines from the F8 to advance to the OYT. The top lines advanced to the OYT based on GEBV are cycled back into the crossing block in order to continue to improve the population. From the OYT, the best performing lines based on phenotype are advanced to the replicated yield trials (RYT), and the best performing lines from the RYT are advanced to the multi-environment trials (MET). Lines from the MET are then selected based on GEBV as parents for the next generation of recurrent selection. Models are built at each stage in which GEBVs are used for selection based on a subset of the lines in the population (~300 individuals representing different families) that are both genotyped and phenotyped to form the training set. The rest of the individuals in the population are genotyped only in order to calculate GEBVs (Spindel et al. 2015)

for model training (Bernardo 2016). While empirical studies are needed to validate these simulation results, they strongly suggest an interesting avenue for applying GS into rice breeding, as in the past, great breeding gains in rice have been made by harnessing exotic variation and alleles (Kovach and McCouch 2008; Xie et al. 2008; McCouch et al. 2013).

A final but vital consideration to incorporating GS into rice breeding programs is that of cost-benefit and resource allocation. The exact costs and benefits of GS vary too much by breeding program to suggest any hard and fast rules regarding when GS should be implemented. It is thus in the hands of individual breeders and, ideally, their geneticist collaborators, to determine when and how it is most efficient to incorporate GS into their programs. However, the following should be taken into consideration. If plant breeders have the option of increasing PS accuracy by adding additional phenotype trials to their program, then the cost of adding a phenotype trial must outpace the cost of the necessary genotyping to implement GS in order for GS to be cost-efficient, or else, the added accuracy from genotyping needs to increase the program's genetic gain more than adding accuracy via additional phenotyping. Thus, the benefit of GS will be highest when (a) seed is limited, when (b) phenotyping is expensive, when (c) GS accuracy is very high, or when (d) intensity of selection can be drastically increased, possibly via rapid generation advance.

Finally, it must be noted that the availability of a genotyping platform that is affordable and cost-efficient, as well as accessible to breeders in terms of bioinformatics expertise, is a prerequisite for implementing GS in any program. For example, it is entirely possible, and not uncommon, for a breeder to wish to implement GS but has no resources to pay for genotyping, even if the benefits of doing so are very clear. Indeed, with the recent and controversial enforcement in the USA of a patent on genotyping-by-sequencing held by Keygene (Petes 2016), and the current prohibitively high costs of fixed arrays for all but the largest breeding programs, the availability of affordable genotyping to small breeding programs could become a logjam for implementing GS in rice.

24.4 Evolving Genomic Selection Technologies

Thus far we have discussed GS models that incorporate genetic effects and phenotypic data in numerous ways, but moving forward into the future, many possibilities exist for incorporating a wider array of biological data and knowledge into GS and quantitative genetics breeding models. This can be thought of as the evolution of genomics-assisted breeding to "biology-assisted breeding" (Buckler 2017). These new "biology-assisted breeding" models could include, but are not limited to, (1) GS models that make use of other "omics" datasets such as transcriptomics data, metabolomics data, proteomics data, or ionomics data, (2) combining GS models with crop growth models, and (3) combining GS with genome editing.

While multiple authors have proposed making use of transcriptomics data in conjunction with genomics-assisted selection (Buckler 2017; Fernie and Schauer 2009; Kadarmideen et al. 2006), as yet, very little work has been published in this area. A recent paper by Kremling et al. (2017), however, provides strong evidence that this approach could be a powerful tool for improving GS prediction accuracies. The authors collected RNA expression data on 299 diverse maize lines and found that using either the expression data of the 5000 most expressed genes or the absolute deviation between expression and population mean of those genes could be used to accurately predict seed weight. This was found to be the case even though the expression profiles were collected in different environments and years than the seed weight phenotypes. Models incorporating other omics datasets could be similarly useful but must be tested empirically. Metabolomics and proteomics models may be particularly useful when breeding for crop nutritive value (Fernie and Schauer 2009).

Because models used in GS usually consider only the relationship between marker genotypes and phenotypes, prediction accuracy decreases when phenotypes are affected by environmental conditions (Resende et al. 2012). This prevents the application of genomic prediction models for predicting the genetic ability in a wide range of environments. For solving this problem, it is necessary to construct a prediction model with environmental data (e.g., day length, irradiation, air temperature, humidity, soil temperature, soil moisture, and soil fertility) as inputs in order to model the relationship among marker genotypes, environmental conditions, and phenotypes. Crop growth modeling is a well-established method to model the relationship between environmental conditions and phenotypes of agronomic traits (Sinclair and Seligman 1996). The method enables us to build a model that predicts phenotypes of agronomic traits under specific environmental conditions and to use the predicted values for cultivation management. Fusing these models with models for GS, we can leverage the relationship among marker genotypes, environmental conditions, and phenotypes of target traits for the selection. Several methods have been proposed for the model fusion (Heslot et al. 2014; Technow et al. 2015; Cooper et al. 2016; Onogi et al. 2016). One of the most direct methods is what builds GS models to predict parameters of a crop growth model. With these models, parameters of the crop growth model can be predicted based on genome-wide marker genotypes, and the phenotypes of target traits under various environmental conditions can be predicted based on the predicted parameters and environmental data. This makes it possible, for example, to predict the performance of lines/plants in various environments before field trials are carried out. In addition, it will be possible to predict what performance is expected for the lines/plants in the future even if climate change progresses. For constructing such a complex system, machine-learning methods may be more flexible and capable of modeling the complexity in the relationship among marker genotypes, environmental conditions, and phenotypes than the fusion of GS models and crop growth models.

Genome editing via the CRISPR/Cas9 system also represents a new avenue for developing improved crop varieties. Unlike GS, developing improved crop varieties using genome editing requires an expert understanding of the genetics

underlying a trait of interest as a specific causal mutation must first be identified and validated before it can be targeted using genome editing. However, once such targets are identified, CRISPR/Cas9 would be an excellent means of rapidly introducing useful new alleles identified from any genotype source into adapted breeding material. GS could then be used on the resulting breeding material to produce new combinations of adapted and gene-edited alleles (Spindel and McCouch 2016).

This type of genome editing + GS breeding method could also make it a simple matter to utilize rice wild relatives and more related germplasm (exotic germplasm) in a rice breeding program for the purpose of expanding the genetic base of breeding materials. Normally, it is challenging and time-consuming to cross adapted to exotic germplasm, and such crosses are traditionally followed by generations of backcrossing to eliminate deleterious alleles and allele combinations present in the exotic germplasm. GS breeding schemas on their own have been demonstrated to make this process more rapid, especially if the beneficial alleles present in the exotic germplasm are known (Spindel et al. 2016; Bernardo 2009, 2014, 2016); however, the most efficient schema would most likely be one in which genome editing and GS are combined.

Phenomics is considered to be one of important "omics" technologies, because phenotyping is now a significant bottleneck of "omics" researches. In genomics-assisted breeding, technologies for high-throughput phenotyping (HTP) are important to assess the association between "omics" data and phenotypes. HTP enables the measurement of phenotypes in large scale and high density across time at high accuracy and low labor intensity (Furbank and Tester 2011) and thus suitable for preparing a large dataset for building GS prediction models. HTP allows us to assess detailed features of plants beyond the trait breeders and researchers used to focus on and to boost the improvement of genetic ability of plants (Ledford 2017). As suggested by Rutkoski et al. (2016), HTP is useful to measure secondary traits that increase the accuracy of genomic prediction of a target trait. In rice, HTP using ultrasonic sensor, infrared thermometer, and multispectral sensor enabled to measure a large population nondestructively and in a fraction of the time and to identify genomic regions controlling yield and yield components (Tanger et al. 2017). HTP has been used for evaluating salinity sensitivity of rice during growth stage, and specific time intervals that characterize the early responses of rice to salinity were identified (Al-Tamimi et al. 2016). Among various HTP methods, remote sensing of plant canopy using low-cost unmanned aerial vehicle will be one of the efficient and routinely used methods, as suggested by current reports (Shi et al. 2016; Haghighattalab et al. 2016; Tattaris et al. 2016; Watanabe et al. 2017). One of the major challenges in HTP is the measurement of root traits, because they are important traits related to drought stress tolerance and nutrient absorption ability but currently not easy to measure (see also Chap. 14).

The implementation of genomic selection in plant breeding will accelerate the accumulation of "omics" data of breeding populations. The accumulated data will be useful not only for building genomic prediction models but also for dissecting genetic mechanisms of complex traits. Biological knowledge revealed by the

dissection of complex traits will be useful for improving the accuracy of GS. In genomics-assisted breeding, genomics and breeding will be both rewarded through mutual reinforcement (Poland 2015).

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Chapter 25
Genome-Wide Association Mapping of Complex Traits in Rice

Xuehui Huang and Bin Han

Abstract In rice varieties, there are many naturally occurring genetic variations. Most of morphological, developmental, and physiological variations in rice belong to complex quantitative traits controlled by dozens of genetic variants. One of the most important aims in rice genetic studies is to identify individual genes and their allelic mutations underlying some phenotypic variations in rice through the way of genetic mapping. In this chapter, we begin by addressing the potential difficulties in genetic dissections of complex traits. We then discuss recent progresses on high-resolution quantitative trait locus mapping and genome-wide association study in rice. Finally, some prospects in the future to enhance the mapping power and resolution of complex traits in rice are discussed.

Keywords Rice · Complex traits · Germplasm resources · Genetic mapping · Next-generation sequencing · Genome-wide association

25.1 What Are Complex Traits in Rice?

Rice (*Oryza sativa* L.) has a large geographic distribution across the world and contains a large number of accessions available in public germplasm seed banks (e.g., the International Rice Genebank at IRRI). The diverse accessions show numerous phenotypic differences, severing as valuable resources in rice breeding. In these rice accessions, most agronomically important phenotypes (e.g., plant height, flowering time, grain yield, grain quality, and stress resistance) are not

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monogenic traits that are simply controlled by variation of a single gene, but quantitative traits resulting from both variation within multiple genes and complex interactions with environmental factors.

Most agronomic traits belong to complex traits, and the complex genetic architecture with the traits makes that different rice varieties show diverse phenotypic performance in the field. Flowering time (also called heading date in rice) is a typical example for explaining genetic variation of complex traits in rice. When planting 950 diverse rice accessions in the same condition, we found abundant phenotypic differences in flowering time, ranging from 52 to 158 days (Huang et al. 2012). Moreover, the pattern of flowering time variation of the accessions would be dramatically changed when the planting geographic location is changed – the correlation coefficient (r^2) of flowering time of 1495 hybrid rice accessions between short-day condition of tropics region and long-day condition of subtropics region was very low (~0.1; Huang et al. 2015). Genetic analysis has identified several major-effect genes underlying this variation in rice, including *Hd1*, *Hd2*, *Hd3a*, *Hd6*, *Hd9*, *Ehd1*, *Ehd2*, *Ghd7*, *Ghd8*, and *DTH2* (Matsubara et al. 2014). Diverse combinations of allelic variation in these flowering time genes (coupled with some minor-effect genes to be characterized for flowering time in rice) generate the visible genetic differences of thermo-sensitivity and photosensitivity in diverse rice accessions (Summerfield et al. 1992).

Of note, flowering time in rice is a morphologically distinguishable quantitative trait that could be phenotyped easily and accurately. Besides, this trait "flowering time" is generally of very high heritability (~90%) thus greatly facilitating all kinds of genetic analyses. However, the situation is not so simple for many other traits. Many traits related to grain quality and stress resistance cannot be observed or counted manually, while many yield components in farmland appeared a relatively low heritability, making genetic studies on these traits more complex.

25.2 Has Genetic Mapping of Complex Traits Become Possible?

In order to understand the molecular bases of complex traits, rice researchers aimed to pinpoint the exact causative gene or even the causative variants controlling the phenotypic variation (Yamamoto et al. 2009). However, it is very challenging to catch the causative genes directly from so many genes (~40,000) and intraspecific genetic variants (at least several millions) in rice genome, especially for those genes with modest or minor genetic effect on phenotypic variation. Generally, genome-wide association study was performed to localize several genomic regions controlling one trait (often called as quantitative trait loci) and learn about their phenotypic effects underlying the trait firstly, and other follow-up works (e.g., fine mapping, coding sequence analysis, transgenic tests) were used to discover the key genes in the local region.

The high-quality rice reference genome sequence and various genetic approaches now allow the analysis and understanding of genetic variation of the complex traits at the nucleotide sequence level. With the release of a map-based sequence of the rice genome for the cultivar Nipponbare (*Oryza sativa* ssp. *japonica*) (International Rice Genome Sequencing Project 2005), the rice scientific community could create a nearly full list of rice genes in 12 chromosomes and get enough information for designing molecular markers (McCouch et al. 2002). Owing to these advents, forward genetic approaches can be used to map the genomic regions and retrieve the candidate genes controlling complex traits variation in various genetic populations.

There are two commonly used genetic approaches for genetic mapping: linkage mapping using biparental recombinant populations and genome-wide association mapping using diverse accessions in natural populations. Most of the genes that have been identified in rice for the genetic basis of complex traits are based on traditional linkage mapping, which usually used two varieties with large phenotypic difference for the target traits as parental lines and a large number of recombinant lines for quantitative trait locus mapping. Until recently, high-throughput genotyping methods (e.g., the second-generation sequencing-based genotyping) have advanced fast-forward rice genetic studies, and genome-wide associations began to become as an alternative approach to investigate the genetic architecture of complex traits.

25.3 Quantitative Trait Locus (QTL) Mapping and Cloning in Rice

Over the last decade, a number of QTLs have been mapped using the linkage map constructed from DNA markers in the rice genome. Typically, two rice accessions with many phenotypic differences (e.g., *japonica* cv. Nipponbare and *aus* cv. Kasalath) were selected firstly for inter-crossing. The resulting F_2 population or recombinant inbred line (RIL) population is genotyped with hundreds of molecular markers and phenotyped with several traits of interest. Due to the marker density and the population size, the mapping resolution in traditional QTL studies was mostly within several megabases, far from gene-level mapping resolution. In addition, the power to identify QTLs with modest or minor genetic effects in QTL mapping strongly depends on the population size and parental combination, because genetic dissection of complex traits is essentially a statistical analysis, in which the greater the population size, the more "statistically significant" the mapping results will be. Hence, high-throughput methods are much needed to simplify labor-intensive experimental procedures thus enabling the rapid genotyping of very large populations, and meanwhile ultra-dense markers in the linkage maps are required to capture the exact recombination events in each recombinant line to improve QTL mapping resolution.

In order to address these limitations from conventional genotyping methods by scoring on agarose gels of PCR markers, some high-throughput methods for high-density genotyping have been developed by utilizing SNP array, genotyping by sequencing (GBS) with restriction enzymes (Elshire et al. 2011), or whole-genome low-coverage re-sequencing. As a proof-of-principle experiment, totally 150 rice RILs from a cross between *indica* cv. 93-11 and *japonica* cv. Nipponbare were used for bar-coded multiplexed sequencing (Huang et al. 2009). A computational method was developed for implementing Bayes-based algorithms and conducting all analyses from aligning raw sequence reads to genotype calling of the full genetic population. With only ~0.02× genome coverage for each rice RIL, a high-density linkage map (at least one informative maker per 100-kb) was constructed for high-resolution QTL mappings by a Bayes-based algorithm for genotype calling in each sliding window across the rice genome, which could yield a genotype calling accuracy of ~99%. Recently, the most updated NGS system has reached the level of throughput as high as 2 Tb (equivalent to 5000-fold rice genome size) per sequencing run in less than 1 week, and the library construction for bar-coded multiplexed sequencing has been greatly simplified due to the application of transposon-mediated DNA library preparation (Picelli et al. 2014). Owing to these technical advances, the method has become more and more cost-efficient and convenient for genome-wide mapping in rice populations. For example, this method helped us to genotype a large number of F_2 lines ($n = 10{,}074$) from 17 rice populations (Huang et al. 2016). Using the high-resolution genotype maps that we obtained, genome-wide mapping of many complex traits was feasible, in which many QTLs, even with modest effects, could be identified with the mapping resolution of approximately 300 kb.

There are usually two steps in linkage mapping of QTLs in rice. The genetic population that is used in the first step often contained hundreds of rice lines, and genome-wide mapping was performed to scan all the QTLs across the rice genome using conventional methods like composite interval mapping. For the complex traits, usually several QTLs could be detected, with various phenotypic effects. In the second step, typically one large-effect QTL with a relatively high likelihood of odds (LOD) value is selected for fine mapping. Because the complex traits are controlled by multiple QTLs, a new population from advanced lines with appropriate genetic backgrounds needs to be developed in the fine mapping procedure to exclude the influence of other QTLs segregating between parental lines of the linkage population. Up to date, the most effective approach in rice is the generation of near-isogenic line (NIL) for the target QTL. That is, F_2 or similar lines were backcrossed with one parental line, followed with intensive marker-assisted selections at each subsequent backcross generation. After several generations of backcrosses and selections, we are able to find the NILs that contained the donor parent's genotype only around the target QTL and the recurrent parent's genotype in other backgrounds. Consequently, the cross between the NIL and the recurrent parent

could be used to generate a very large population (at least thousands of lines) for fine mapping (called NIL-F_2 population), because the effects of all the other QTLs disappear in this specific cross combination (NIL and its recurrent parent). In rice, despite of huge laborious works, large populations from NIL-F_2 appeared to be very powerful in QTL cloning, often narrowing down the causative gene to several kilobases. For the population from the cross between Nipponbare and Kasalath, many NILs have been developed and successfully used, resulting to gene cloning of tens of QTLs underlying many complex traits (e.g., grain shape, grain weight, and phosphorus-deficiency tolerance; Shomura et al. 2008; Gamuyao et al. 2012; Song et al. 2015). Moreover, as an alternative choice, the residual heterozygosity (RH) could be used as well for fine mapping. Based on genome-wide mapping results in the first step, RILs or similar lines can be screened for heterozygotes around the target QTL (Xu et al. 2008; Liu et al. 2009; Bai et al. 2010). Selfing of the RILs with marker-assisted selections gets the opportunity for researchers to find the lines (RH-F_1) exhibiting heterozygosity only around the QTL but is homozygous in all the other genetic backgrounds. Selfing of the ideal lines produced progenies (RH-F_2) segregating for the target QTL similar with NIL-F_2 populations, which has been used in several studies for fine mapping of rice QTLs.

25.4 Genome-Wide Association Studies in Rice

These QTL studies in rice represent to be very powerful and have provided many insights into the natural variation of complex traits (Miura et al. 2011). However, genetic analysis relying on biparental linkage mapping can only identify genetic variation between the two parental lines, that is, only a small fraction of allelic variations among diverse rice varieties. In human genetics, genome-wide association study (GWAS) is the most powerful way to understand the genetic basis of complex traits (e.g., genetic diseases; Altshuler et al. 2008). The first GWAS in human genetics was conducted in 2005. This study used 96 patients with age-related macular degeneration (ARMD) and 50 healthy controls for whole-genome genotyping and identified one candidate gene encoding complement factor H (Haines et al. 2005). The landmark work in the history of GWAS in human genetics was conducted by a large project (the Wellcome Trust Case Control Consortium 2007), which collected 14,000 cases of 7 common diseases (~2000 individuals for each disease) and 3000 shared controls and uncovered many new disease genes underlying these diseases. During the recent 10 years, hundreds of large-scale GWAS have been made in human genetics which have created comprehensive catalogues of SNPs associated with tens of traits.

There are many differences of population character or reproduction style (e.g., inbreeding or outcrossing) between human and rice plants. Rice has its advantage

for GWAS, because in principle the rice panel for GWAS could be genotyped only once and phenotyped many times for kinds of complex traits. The first GWAS in rice was conducted in 2010, which used a number of inbred rice accessions as the GWAS panel (Huang et al. 2010). A haplotype map of rice genome was constructed through whole-genome low-coverage sequencing of 517 rice landraces and *k*-nearest neighbor algorithm-based imputation. Using this panel, the associations between 671,355 common SNPs and phenotypic variation of 14 agronomic traits in 373 *indica* lines were tested and identified several strong association signals including 6 previously identified genes. The loci identified through GWAS explained ~36% of the phenotypic variance. Another GWAS used a custom-designed genotyping array (with 44,100 SNPs) for a global collection of 413 diverse rice varieties (Zhao et al. 2011). Totally 34 morphological, developmental, and agronomic traits were phenotyped, and GWAS demonstrates various genetic architectures underlying these complex traits in rice. GWAS was also used in rice to dissect another kind of traits – plant metabolites. Comprehensive profiling of 840 metabolites was obtained in 529 rice diverse accessions. A further metabolic GWAS based on ~6.4 million SNPs identified complex and distinct genetic regulation of metabolites (Chen et al. 2014). With the candidate genes identified for genotype-phenotype associations, follow-up experiments by functional genomics approaches will be needed to validate whether the detected candidates are the causative genes of the phenotype (see the proposed approaches in the last section of the chapter).

Although most traditional rice landraces and most modern cultivars of *japonica* subspecies are inbred lines with whole-genome homozygous genotypes, there are a large number of *indica* cultivars belonging to hybrid rice. The tool of GWAS can also be used in the varieties of hybrid rice (F_1 lines with immortalized genotypes). Using 1495 diverse hybrid rice accessions, the genetic architecture of 38 traits including grain yield, grain quality, and disease resistance was analyzed using GWAS (Huang et al. 2015) which identified 130 associated loci underlying these agronomic traits (including 53 loci with well-characterized genes and 77 novel associations). The information of the associated SNPs has direct implications for rice breeding.

Rice (*O. sativa*) contains two major subspecies: *indica* rice and *japonica* rice. The population differentiation statistic (F_{ST}) between the two subspecies was calculated to be ~0.55 when using whole-genome resequencing data. The value (0.55) suggests that rice have a very strong population structure (Reig-Valiente et al. 2016). Population stratification (also referred to as population structure) in rice is a serious problem for GWAS, because many associations result from the underlying structure and not a causative locus, making the occurrence of a large number of false positives. In other words, the genotypes from different genomic loci, no matter the loci are close together on a chromosome or even distributed on different chromosomes, are tended to be correlated. Given a very clear population differentiation between the two subspecies, in most cases we look for associations for the *indica* population and the *japonica* population separately (Huang et al. 2012). However, even within each subspecies, there exists population structure, where

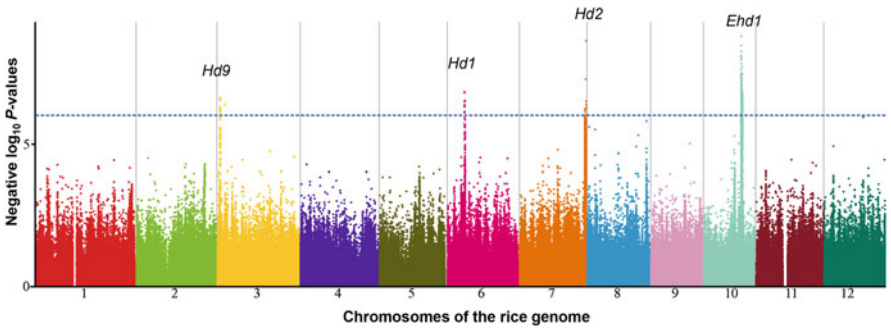

Fig. 25.1 The Manhattan plots of GWAS for heading date in rice using the method of LMM. Negative $\log_{10}$ *P*-values from linear mixed model (y-axis) are plotted against SNP positions (x-axis) on each of 12 rice chromosomes. The genome-wide significant *P*-value threshold (10^{-6}) is indicated by a horizontal dash-dot line. The loci with well-characterized genes are indicated near the association peaks

the photoperiod and temperature clines may have been the primary factors of the population structure. Fortunately, several statistical methods have been developed to correct these confounders including population structure, family structure, and cryptic relatedness. The linear mixed model (LMM) uses the measure of genetic similarity (e.g., the kinship matrix) for the corrections (Yu et al. 2005; Kang et al. 2010; Zhang et al. 2010). The method generally works well in rice GWAS and can partially solve the problem of modest population structure (Fig. 25.1). To perform GWAS more efficiently, in addition to the application of statistical corrections, it is important to select a panel that is not genetically highly structured yet exhibits high phenotypic diversity. For example, 176 *japonica* rice accessions developed by breeding programs in Japan were selected, and the careful experimental design using the closer lines in the *japonica* panel enabled more effective GWAS analysis (Yano et al. 2016).

25.5 "Next-Generation Populations" for Genetic Mapping

The segregation genetic populations (e.g., F_2 and RIL) have three genotypes present with the proportion of 1:2:1 (in F_2) or 1:1 (in RIL) and uniform distributions of allelic combinations between different loci. However, in natural population for GWAS, the allelic variations are quite unevenly distributed, with strong bias in allele frequencies. Large-scale re-sequencing has demonstrated that most genetic variants identified in natural population are rare or of low frequency in both plants and animals. For example, in case of human, approximately 700 height-associated SNPs have been identified through GWAS so far, explaining totally 23.3% of height heritability (Marouli et al. 2017). Many of the known height-associated SNPs are of low frequency, and the remaining associated SNPs to be identified

for the rest of height heritability are probably rarer. For GWAS, it is very difficult to identify associations from rare alleles by statistical methods. Moreover, in case of rice, diverse accessions with a high level of genetic diversity often contain multiple alleles at the same gene with differentiated genetic effects, making GWAS and related genetic analysis very complicated. In summary, the strategy of biparental linkage has the limitations including low genetic diversity and low mapping resolution, while the strategy of GWAS has the limitations including population structure and low power to map rare allele and multiple alleles, really difficult balance to strike between them (Nordborg and Weigel 2008; Myles et al. 2009).

One of the ways to improve the power of genetic mapping is the generations of "next-generation populations" through careful experimental designs. Constructing collaborative recombinant populations selected from multiple diverse parental lines can help to control for population structure, take advantage of both historic and recent recombination events, as well as identify alleles with low frequency in the population. The nested association mapping (NAM) population designed by Edward Buckler, James Holland, and Michael McMullen (McMullen et al. 2009) has been developed for identifying and dissecting complex traits in maize (*Zea mays*). The NAM population consisted of 25 RIL populations. The NAM founder lines were carefully selected from a larger panel of diverse maize accessions to maximize diversity. Each inbred line was crossed with the common reference parent B73 (used in maize reference genome sequencing), creating ~200 RILs for each subpopulation. Joint analysis of the full population with 5000 lines enabled a powerful dissection of complex traits in maize including flowering time, leaf architecture, and leaf blight resistance (Buckler et al. 2009; Tian et al. 2011; Kump et al. 2011; Poland et al. 2011). Some similar designs have been performed in *Arabidopsis* (e.g., the MAGIC lines and AMPRILs; Kover et al. 2009; Huang et al. 2011).

In rice, few studies using multiple collaborative populations for joint analyses have been reported, probably due to partial reproductive isolation between subspecies. Wide-compatibility genes in rice could be used to generate *indica-japonica* crosses more conveniently. For example, the gene *S5*, a major reproductive barrier regulator, has been identified in the cultivated rice (Yang et al. 2012). The wide compatibility allele of *S5* could further facilitate inter-crossings and the constructions of genetic populations between *indica* and *japonica*. Similar with the design in maize and *Arabidopsis*, diverse rice accessions with maximized diversity could be selected from rice germplasm collections according to the phylogenetic relationship from whole-genome variation data. Dozens of diverse accessions that were selected can be crossed with one to two common reference parents (e.g., Koshihikari and 9311) to generate backcross inbred line (BIL) populations (e.g., the lines of BC_4F_2). The panel with multiple BIL populations collectively will be useful for both breeding and complex trait mapping (Fig. 25.2). With the continuing improvement of NGS systems and the rapid decrease in the costs, genome-wide mapping using these "next-generation populations" will become a powerful tool to dissect the causal loci that account for complex traits in rice.

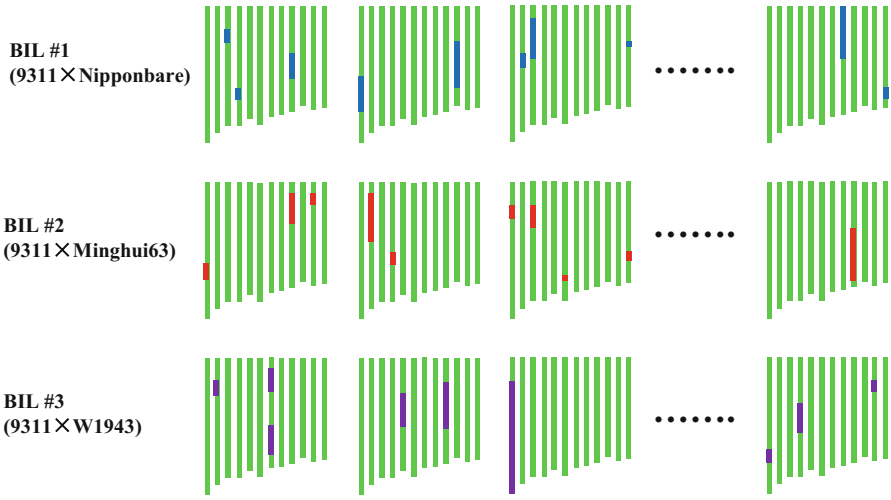

Fig. 25.2 Construction of multiple sets of BIL as next-generation mapping population in rice. The genotypes of *O. sativa* ssp. *japonica* Nipponbare, *O. sativa* ssp. *indica* Minghui 63, and *O. rufipogon* W1943 (as donor parents) are colored in blue, red, and purple, respectively, while the genetic background of *O. sativa* ssp. *indica* 9311 (as common reference parents) is colored in green

25.6 Dissecting Rice Traits for Understanding Heterosis

Genome-wide mapping of yield traits in rice also helps to understand the genetic cause of heterosis. Heterosis, or called hybrid vigor, is the phenomenon that the hybrid (F_1 generation) shows better yield performance than both parents. The efficient use of heterosis has created many high-yielding hybrid rice varieties, serving as one of the most important applications of genetics in agriculture (Cheng et al. 2007; Riedelsheimer et al. 2012). There are some practical difficulties to face in rice heterosis studies: (1) there are diverse hybrid rice combinations to be selected, of which the heterosis phenomenon may be under several independent genetic mechanisms; (2) grain yield in rice is a very complex trait composited with multiple yield components, which makes the genetic dissection to be a big challenge; and (3) epistatic interactions among genes and the distribution of homozygous/heterozygous genotypes may affect the evaluations of heterotic effects. That means deciphering the nature of heterosis will rely on genetic populations of designed cross combinations to use, all yield component traits to be surveyed, and new genetic and genomic approaches to implement for mapping the important genetic contributors in heterosis.

The cross between Zhenshan 97 and Minghui 63 created an elite rice hybrid Shanyou 63 that has been widely cultivated in China since the 1980s. As a representative hybrid combination with strong over-parent heterosis phenomenon, a RIL population between the two parental lines was constructed firstly, and an

"immortalized F_2" population was generated subsequently through random crossing between the well-genotyped RILs. To explain the genetic basis of heterosis in this hybrid, a genetic dissection of yield traits was performed using the "immortalized F_2" population, and the genetic effects of the loci across the rice genome were calculated (Xiao et al. 1995; Hua et al. 2002; Zhou et al. 2012). By analyzing single-locus and digenic genetic effects at the whole-genome level, the relative contributions of different forms (dominance, overdominance, and epistasis) in heterosis were able to be estimated, and the results showed that the relative contributions of the genetic components varied with traits.

In the study for rice heterosis, totally 10,074 F_2 lines from 17 representative elite hybrid rice were generated, genotyped, and phenotyped (Huang et al. 2016). Through both large-scale QTL mapping and GWAS, the key genes and genomic loci contributing to yield heterosis were identified at high resolution, and their phenotypic effects were evaluated using the large genetic data. It was observed that the heterozygous state of the heterosis-related genes (e.g., *hd3a* gene controlling flowering time and *TAC1* gene controlling tiller angle; Kojima et al. 2002; Yu et al. 2008) generally acts through the way of partial dominance for each yield-related component but over-parent heterosis for overall performance when grain yield, growth time, and plant density are all considered.

25.7 Catching Up Agronomic Trait Genes (CATG) Through Integrated Approaches

Both linkage mapping and association mapping rely on recombination events to narrow down the causal gene. The GWAS panels for outcrossing organisms, such as human or maize, generally contain a large number of historical recombinations. Hence, the resolution of human GWAS or maize GWAS in most cases can reach the gene level (Li et al. 2013). As an inbreeding species, most genetic populations in rice only have limited recombination events, leading to the major limitation in rice GWAS – the mapping resolution mostly reach the level of approximately 100-kb, no matter conventional natural populations or "next-generation" populations are used. The 100-kb region usually contains several annotated genes. For the goals like phenotype prediction and molecular breeding, this resolution is already high enough (Riedelsheimer et al. 2012). Using the associated SNPs or the haplotype information in the local region, the advantageous alleles could be selected from the germplasm collections, and marker-assisted gene pyramiding could be performed to create ideal genotypes. However, for functional characterization of rice genes, the causal genes should be pinpointed from several candidates (Zhang et al. 2008), where other omic approaches and the tools of molecular biology are needed (Lu et al. 2010).

In one follow-up study of rice GWAS, gene expression information was used to catch the causal gene. GWAS in 381 *japonica* has localized the position of a QTL *GLW7* controlling grain size into a linkage disequilibrium (LD) block (Huang et al. 2012). Rice has relatively long LD decay, which makes it difficult to identify the causal gene (Mather et al. 2007). To resolve this problem, the expression pattern analysis of candidate genes was integrated, and their knockout lines (T-DNA mutants) were used. Several large-grain and small-grain *japonica* varieties were randomly selected to measure the expression levels of all the genes at the local region in panicles, leaves, and roots. Of them, all genes showed no difference in expression levels in panicles except *OsSPL13*. Furthermore, through the experiments including T-DNA mutants and genetic transformation, it was confirmed that *OsSPL13* is a causal gene for *GLW7* that was a key element in producing larger grains and panicles and eventually improved grain yield in cultivated rice (Si et al. 2016). The combination approach succeeded to overcome defects in GWAS of rice.

In another GWAS, the functional genetic variants were deeply investigated to determine the strong candidates (Yano et al. 2016). That is, although many polymorphisms were included in a candidate region, the functionally meaningful polymorphisms were less numerous. After detecting significant signals through GWAS with individual nucleotide polymorphisms, a few strong candidates could be found based on the function of polymorphisms and annotation information including that of *Arabidopsis* homologs. In sesame GWAS, the integration of genetic variant annotation, expression profiles, and known metabolic or signaling pathways showed very effective, and 46 candidate causative genes were identified (Wei et al. 2015). Hence, the integrated approach, calling CATG here, will be an important step following genome-wide mapping (Fig. 25.3). As a model plant, rice has its unique advantage since many databases including kinds of informatics resources are publically available. For example, more reference genome sequences in rice through deep sequencing and whole-genome de novo assembling and more RNA-Seq data of various tissues under different stresses will be finished, which can greatly aid in the detection of causal genes and variants.

In the future, the improvement of genome-editing technologies and synthetic biology approaches will be able to rapidly create knockout or knockdown lines of candidate genes, and novel genotypes in rice, and will certainly help to validate the causative genes after genetic mapping. Moreover, a scientific team has developed a way to use CRISPR/Cas9 to mimic mitotic recombination in yeast cells, which lead to a new way to map out genes and determine their functions (Sadhu et al. 2016). Once the method can be used in rice genome to build mapping panels with targeted recombination events, the technical revolution will allow us to rapidly and systematically identify causal variants underlying complex traits.

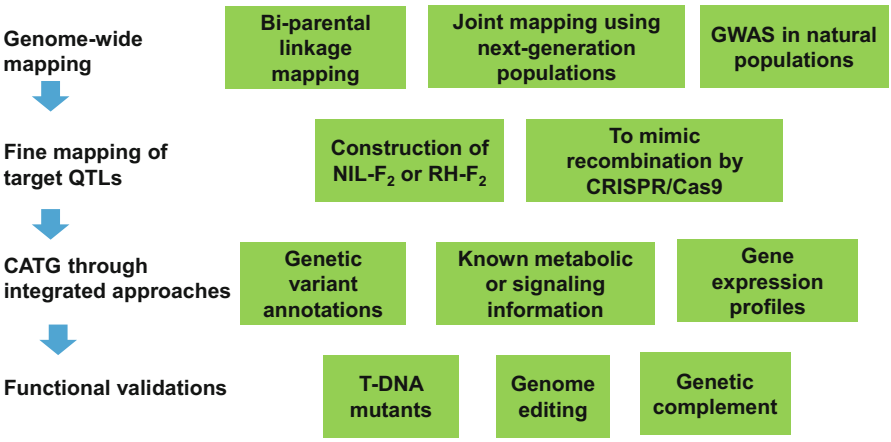

Fig. 25.3 A schematic view of genome-wide mapping and causative gene identification underlying the phenotypic variation of complex traits in diverse rice varieties

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Chapter 26
Next-Generation Breeding of Rice by Whole-Genome Approaches

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Abstract Owing to recent developments in DNA sequencing technologies, whole-genome analysis of crop species has become a routine procedure. In order to improve local rice cultivars adapted to northern Japan, we are applying whole-genome analysis to identify and utilize useful alleles for crossbreeding. Here we show that large-scale generation of genetic resources combined with whole-genome analyses including MutMap and QTL-seq provides a powerful platform for the application of "next-generation breeding" in rice.

Keywords Whole-genome sequencing (WGS) · Mutant · Recombinant inbred lines (RILs) · MutMap · QTL-seq

26.1 Introduction

Rice is the principal staple crop in Asia including Japan. The Tohoku region of northern Japan that also includes Iwate Prefecture is the major rice production area in the country. Iwate Biotechnology Research Center (IBRC), in collaboration with Iwate Agricultural Research Center (IARC), has a mandate for basic researches geared toward the improvement of elite local rice cultivars. The main mission of IBRC and IARC is the delivery to local farmers of high-yielding and quality rice cultivars that are competitive on the market, as well as resistant to various biotic and

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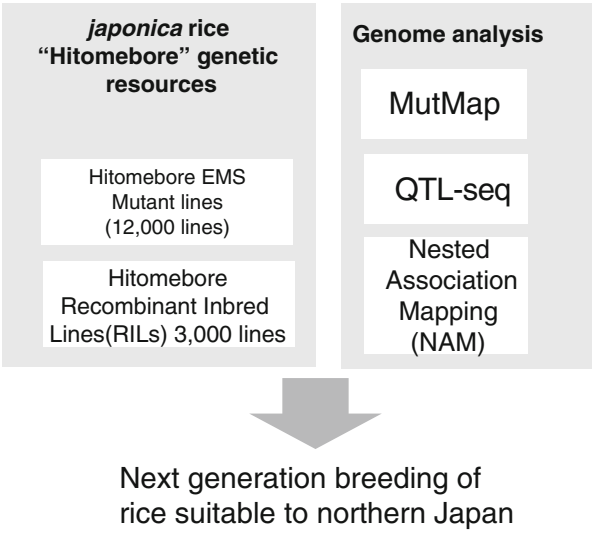

Fig. 26.1 General scheme of rice "next-generation breeding" (Adopted by IBRC and IARC)

abiotic stresses such as blast disease and too cold summer. To meet this goal, we are employing crossbreeding to improve these elite local rice cultivars. Our approach, which is different from the practices of conventional breeding commonly employed, involves a quick and precise breeding based on genome analysis that targets traits of interest. We call this approach "next-generation breeding." To this end, we have devoted considerable efforts to (1) establishing large number of rice genetic resources to identify and exploit useful phenotypic traits and (2) developing genome analysis pipelines that enable rapid identification of alleles that confer the desired traits (Fig. 26.1). In this chapter, we would like to share our experiences and achievements from these on-going endeavors.

26.2 Rice Cultivar "Hitomebore" Adapted to Northern Japan

The most popular variety currently grown in Iwate is a temperate *japonica* rice cultivar known as "Hitomebore." It was developed at Furukawa Agricultural Research Station, Miyagi Prefecture in Tohoku region from a crossbreeding between "Koshihikari," currently the most widely grown cultivar in Japan, and "Hatsuboshi." It was registered in 1991 but was widely adopted after 1993 following the severe cold summer that resulted in extremely low yields in rice including "Sasanishiki," which was the most popular cultivar widely grown in Tohoku region at the time. "Hitomebore" is characterized by strong cold tolerance and excellent eating quality (Table 26.1). These combined with traits that make it suitable for cultivation lead to its wider adoption by farmers over considerable areas in northern Japan. The government census published in 2010 reveals that in terms of total area

Table 26.1 Characteristics of "Hitomebore"

Eating quality	Good
Grain appearance	Good
Cold tolerance at booting stage	Strong
Seedling vigor	Medium
Blast resistance	Medium
Lodging resistance	Weak to medium
Yield	Medium

of production in Japan, "Hitomebore" is second only to "Koshihikari" (http://www.maff.go.jp/j/tokei/sokuhou/syukaku_suitou_09/).

26.3 Whole-Genome Sequence of Rice

The first draft genome sequences of rice were published in 2002 for *japonica* (Goff et al. 2002) cultivar Nipponbare and *indica* cultivar 93-11 (Yu et al. 2002), respectively. Following to these, a high-quality whole-genome sequence was published using a *japonica* cultivar Nipponbare (IRGSP 2005), providing one of the most accurate sequences available for a crop species so far. The "Nipponbare" genome sequence is 389 Mb in size and has set the stage for application of genomics to rice improvement since its release. Useful database and analysis tools are available from Rice Annotation Project Database (RAP-DB) portals (http://rapdb.dna.affrc.go.jp).

26.4 Genetic Resources for Rice Improvement

We have been working over a decade to generate genetic resources that can be utilized for improvement of local rice cultivars. We have generated two categories of resources: mutant lines and recombinant inbred lines (RILs), both in "Hitomebore" genetic background.

26.4.1 Mutant Lines

We have generated a total of 15,000 ethyl methanesulfonate (EMS) mutant lines including 12,000 Hitomebore and 3000 Sasanishiki mutants. EMS is an alkylating chemical that induces primarily GC → AT transition-type nucleotide substitutions. Details of the mutagenesis procedure have been reported elsewhere (Rakshit et al. 2010). Briefly, we treated immature embryos of rice with 0.175% EMS solution immediately after anthesis by immersing flower spikes in a plastic bag containing

EMS solution overnight. This method was adopted in order to minimize chimerism, which occurs more frequently in seed mutagenesis. After mutagenesis, matured seeds of the M1 generation were sown, and plants were grown to set flowers, which were self-fertilized to obtain M2 seeds. Ten M2 seeds per M1 line were planted in paddy field to observe their phenotypes. The majority of induced mutations is heterozygous in the M1 generation and brought to homozygous state in the M2 generation. Accordingly, phenotypes of recessive mutations are expected to manifest in one fourth of the M2 individuals. The chance that we obtain recessive homozygous plants among ten M2 individuals is $1-(3/4)^{10}) \approx 0.94$, which allow us to observe majority of the mutant phenotypes in the M2 generation. We re-sequenced "Hitomebore" and identified a total of 128,704 polymorphic sites by aligning "Hitomebore" short reads to the "Nipponbare" reference genome sequence. We then replaced "Nipponbare" nucleotides with "Hitomebore" ones at all the polymorphic sites to generate a "Hitomobore" reference genome. Resequencing of more than 20 independent mutant lines revealed that our mutagenesis on average induced 1500 SNPs per genome per line. This suggested we can expect 46 SNPs per 1000 bp randomly selected genomic region if we screen the entire 12,000 "Hitomebore" mutant lines. This suggested that the majority of genes were targeted by our mutagenesis. These mutant lines have been used for forward genetics as in MutMap analysis (Abe et al. 2012) described below, as well as for TILLING (Till et al. 2003) that represents a reverse genetics approach (Rakshit et al. 2010).

26.4.2 Recombinant Inbred Lines (RILs)

To exploit the natural variation in rice for breeding, we generated RILs using "Hitomebore" as the common parent. RILs are generated by crossing two lines to obtain an F1 progeny, which is then self-fertilized (selfed) to produce F2 progeny. Each F2 individual is treated as a line, and a single F3 seed derived from selfing of F2 is grown to obtain F4 seeds. This process is repeated several times to generate F7–F9 generations (Fig. 26.2). Finally, a large number of seeds are harvested from each line in the advanced generation. RILs offer two major advantages over F2 population in QTL mapping: (1) Majority of the genome is brought to homozygous state in RILs by repeated selfing, meaning the phenotypes are determined by the additive effects of genes with a minimal influence from dominance effects. This increases the precision of QTL mapping. (2) Each F2 genotype is represented only by one individual, whereas each line of RIL has >1000 seeds with practically an identical genotype. This allows the planting of a large number of individuals to perform phenotyping in replicates, resulting in the reduction of phenotypic variance caused by the nongenetic causes. We crossed "Hitomebore" to 22 rice accessions (8 *aus*, 5 *indica*, 4 temperate *japonica*, 5 tropical *japonica*) presumably representing the *O. sativa* genetic diversity. Approximately 300 F2 seeds per cross were used as the starting materials for generating RILs by single-seed descent (SSD) method.

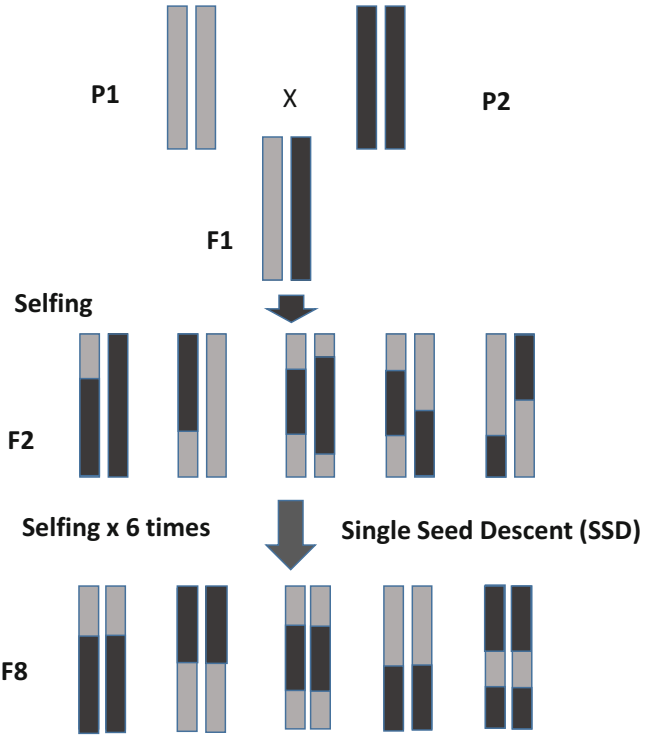

Fig. 26.2 A simplified scheme for the generation of recombinant inbred lines (RILs)

This generated a total of 3000 RILs of F7–F9 generations, with progeny number in each cross ranging from 30 to 300. These RILs have been planted in IARC paddy field and are being used for QTL-seq (see below) and nested association mapping (NAM) studies (Yu et al. 2008).

26.5 Genome Analysis Methodologies

26.5.1 MutMap

In order to exploit "Hitomebore" mutant lines in rice breeding, we developed MutMap (Abe et al. 2012), which is a whole-genome sequencing (WGS)-based method for the identification of mutations responsible for phenotypes of interest. In MutMap, we cross a mutant of interest to the original line used for the mutagenesis. In our case, a mutant is crossed to a "Hitomebore" wild-type (WT) plant. The resulting F1 is selfed to obtain F2 progeny segregating for the WT and mutant-type progeny in a 3:1 ratio provided that the causative mutation is recessive. This crossing scheme is different from the conventional trait mapping in which a mutant

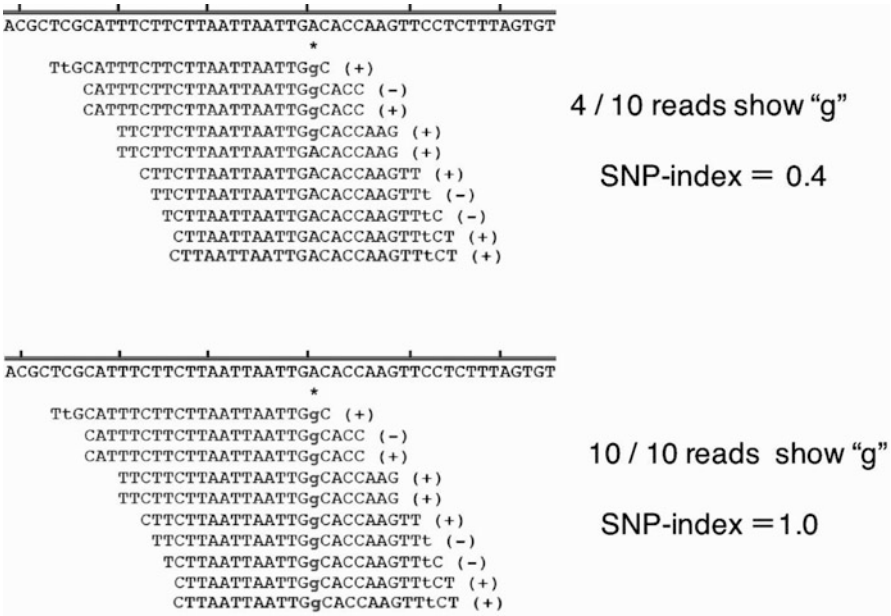

Fig. 26.3 Alignment of short reads to reference genome sequence and definition of SNP-index values

line of interest is crossed to a genetically distant line. The crossing scheme of MutMap allows researchers to target subtle quantitative differences in mutant phenotypes that are difficult to address using distant crosses. DNAs of multiple mutant F2 progeny (usually 20 individuals) are pooled to make a DNA bulk, which is subjected to WGS by the illumina sequencing platform. The resulting short sequence reads of 100–150 bp are aligned to the reference sequence of "Hitomebore." Among the population of F2 progeny showing the mutant phenotype, the frequency of mutated allele at the causative gene is 100%, whereas that of mutations in the genomic regions not related to the mutant phenotype is expected to be 50%. The alignment of short reads obtained from bulked DNA to the reference genome sequence reveals the allele frequencies of all SNPs across the genome (Fig. 26.3). When short reads are aligned to the reference genome, each nucleotide position is usually covered by multiple reads. Here we introduce a measure named SNP-index. Within ten short reads that are aligned to a given genomic region, if all reads had a SNP at a particular nucleotide different from the reference nucleotide, the SNP-index is $10/10 = 1.0$. On the other hand, if four of the reads have SNPs and the rest have the nucleotide identical to the reference nucleotide, SNP-index is $4/10 = 0.4$. This SNP-index is in fact the frequency of particular SNPs among the

population of pooled individuals. Next, we plot a graph relating genomic position of an SNP on the x-axis and its SNP-index value on the y-axis. As explained above, the SNP-index value of the causative SNP responsible for the mutant phenotype should be 1, and those of SNPs on the chromosomes that do not contain the causative SNP are 0.5. However, SNP-index values of the SNPs tightly linked to the causative SNP show higher SNP-index values (0.5< SNP-index <1) due to linkage drag. Therefore, the graph typically shows a cluster of SNPs with higher SNP-index values near the causative one, allowing a rapid identification of causative mutation. The result of a typical MutMap analysis is given in Fig. 26.4. As extensions of the MutMap methodology, we have developed MutMap-Gap (Takagi et al. 2013a) and MutMap + (Fekih et al. 2013).

26.5.2 QTL-Seq

To identify quantitative trait loci (QTL) controlling phenotypic differences between two cultivars, we developed a WGS-based method named QTL-seq (Takagi et al. 2013b). This method is suitable for identification of QTL responsible for natural variation of phenotypes. In QTL-seq, two cultivars are crossed and segregation of phenotypes is observed either in F2 progeny or RILs. The majority of quantitative traits are controlled by multiple genes. Accordingly, the frequency of phenotypes among F2 progeny or RILs typically follows the normal distribution. We then focus on the progeny with extreme phenotypes, those with the minimum and maximum phenotypic values. DNAs of 10–20 progeny with the minimum values are pooled to make the low (L) bulk, and those with the maximum values are pooled to make the high (H) bulk. The L and H bulk DNAs are separately subjected to WGS, and the resulting short reads are aligned to the reference genome sequence generated for either of the parents used for crossing. For each SNP present between the two parents, we obtain SNP-index values and generate a graph relating SNP position and SNP-index as explained above in MutMap. If we use the genome sequence of the parent 1 as the reference, alignment of short reads only derived from the parent 1 genome gives SNP-index value = 0, whereas alignment of reads only from the parent 2 genome gives SNP-index value = 1. Genomic regions not related to the selection of phenotype between L and H are expected to be transmitted equally from the two parents, bringing the SNP-index values to 0.5. The genomic regions responsible for the L and H separation of phenotypes should have SNP-index that significantly deviates from 0.5. Typically, we observed a peak of SNP-index for either of the two bulks (i.e., L bulk) and a valley of SNP-index in the other bulk (H bulk) at the identical genomic position. This identifies the location of QTL controlling the difference of phenotype between the two parental cultivars. A typical result of QTL-seq analysis is shown in Fig. 26.5.

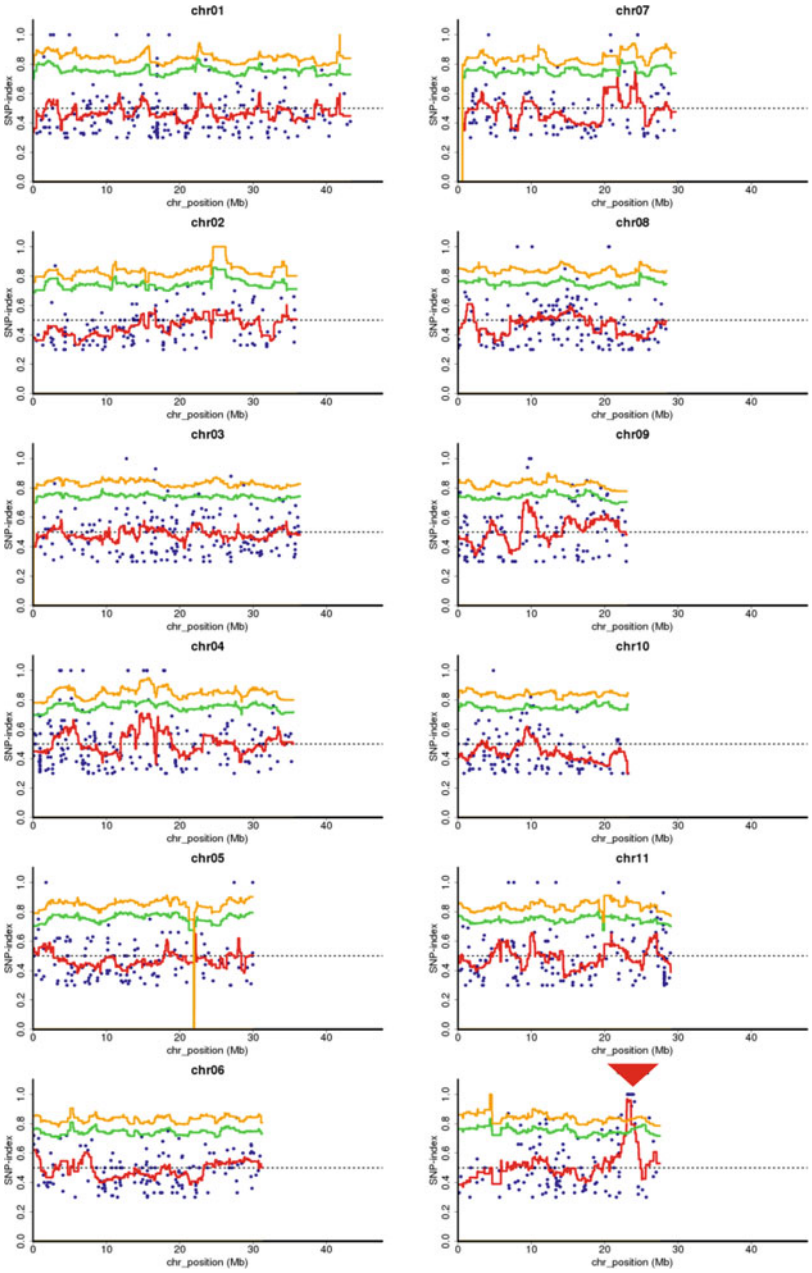

Fig. 26.4 SNP-index plot of the rice 12 chromosomes generated by MutMap analysis. A candidate region in chromosome 12 is indicated by red triangle. Blue dot, SNP; red line, sliding window average of SNP-index; and green and orange lines, sliding window average of 95%- and 99%-confidence level, respectively, under the null hypothesis

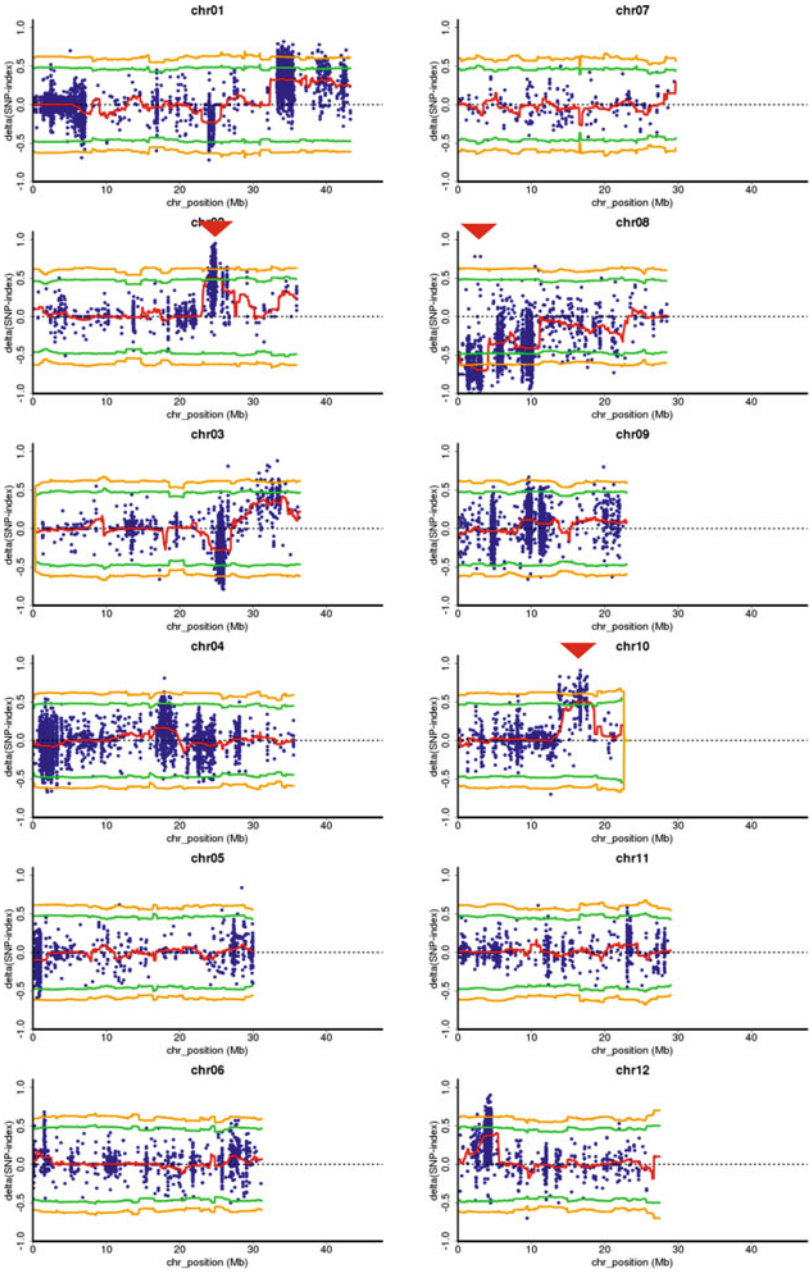

Fig. 26.5 QTL-seq result. These graphs show SNP positions (x-axis) and delta-SNP-Index values (y-axis) deplcting the difference in SNP-Index values between the H- and L-bulks. Three candidate QTLs are indicated by red triangles. Blue dot, SNP; red line, sliding window average of SNP-index; and green and orange lines, sliding window average of 95%- and 99%-confidence intervals, respectively, under the null hypothesis of no QTL

26.6 Examples of Next-Generation Breeding of Rice

26.6.1 Salt-Tolerant Cultivar "Kaijin"

Following the devastating 2011 earthquake and tsunami that attacked Tohoku area of Japan, >20,000 ha of rice paddy field facing the Pacific coast was inundated with seawater, resulting in salt contamination of agricultural land. As the local rice cultivar "Hitomebore" is not tolerant to salt stress, we set out to develop a salt-tolerant rice cultivar in "Hitomebore" genetic background. Accordingly, we carried out a genetic screen for salt tolerance using 6000 EMS mutant lines of "Hitomebore" and identified a mutant that survived 1.5% NaCl supplied to the soil with irrigation water for 7 days. This candidate mutant line was designated as *hitomebore salt tolerant 1* (*hst1*).

To identify the mutation responsible for the high salinity tolerance of *hst1*, we applied MutMap. F2 progeny derived from a cross between *hst1* and "Hitomebore" WT was treated with water containing 0.75% NaCl. The progeny segregated in a 133:54 ratio for salinity-susceptible and salinity-tolerant phenotypes, respectively, conforming to a 3:1 segregation ratio that indicated the salinity tolerance of *hst1* is conferred by a single recessive mutation. DNA was pooled from 20 individuals that showed salinity tolerance and applied to whole-genome resequencing using illumina DNA sequencer. The causative SNP at the nucleotide position 4,138,223 on chromosome 6 corresponded to the third exon of the Os06g0183100 gene, which is predicted to encode a B-type response regulator designated as OsRR22. We only required a year to identify the causative SNP conferring the high salinity tolerance of *hst1* by MutMap.

To develop a salinity-tolerant cultivar that has the *hst1* mutation, we backcrossed "Hitomebore" to *hst1*. After two backcrossing events followed by two consecutive selfings (BC1F3), combined with confirmation of inheritance of the recessive *hst1* allele by Sanger sequencing, we developed a line named "Kaijin" (Neptune in Japanese). "Kaijin" has the same level of salt tolerance as *hst1*, and whole-genome resequencing revealed that "Kaijin" differed from Hitomebore WT by only 201 SNPs, which is a significant reduction from the 1088 homozygous SNPs in the *hst1*. "Kaijin" is practically equivalent to the elite cultivar "Hitomebore" in all agronomic traits except for the salinity tolerance (Takagi et al. 2015).

The example described above has demonstrated the fact that screening of mutant lines combined with MutMap allows the accelerated breeding of cultivar with desirable traits. This is a powerful tool that is now at the disposal of breeders, allowing them to respond to immediate and pressing demands such as the ones caused by global climate changes or natural as well as mandate disasters.

26.6.2 Good Eating Quality Cultivar "Konjiki-no-kaze"

Eating quality of rice determines consumers' preferences and market price. Non-glutinous temperate *japonica* rice cultivars are consumed as staple in Japan, where soft and slightly sticky rice is generally preferred. Amylose content of rice grain's starch is an important factor that affects the eating quality. Therefore, fine-tuning of amylose content is one of the most important targets in rice breeding program in Japan. The gene encoding granule-bound starch synthase I enzyme (GBSSI), named *Waxy* (*Wx*), is required for amylose synthesis in rice. Several *Wx* mutants have been used to breed rice lines with low amylose content, resulting in the release of several cultivars (e.g., "Milky Queen," "Yumepirika"). However, these *Wx* mutant alleles are not suitable as genetic resources to use in Tohoku region because these alleles make grain amylose much lower than the optimal range (14–17% amylose content) that is generally preferred by consumers.

To identify a novel genetic resource of low amylose content suitable for Tohoku region, we carried out a screen of 1600 EMS mutant lines of "Hitomebore" and identified a mutant line "Hit1073" with amylose content lower by only 2.5% compared to "Hitomebore" (17–19%). To identify the causative mutation responsible for the slightly low amylose content, we applied MutMap and succeeded in detecting a few candidate SNPs associated with low amylose content in a genomic region different from that of *Wx*. Using one of these SNPs as a DNA marker, we performed marker-assisted selection (MAS) to the F2 progeny derived from the cross between Hit1073 and Hitomebore WT. Finally, we developed a new cultivar named "Konjiki-no-kaze" (gold-colored wind). This cultivar received a high rating in eating quality that has resulted from slightly low amylose content. We expect this cultivar to be widely planted by farmers in Iwate Prefecture in the near future.

26.7 Prospects

Rapid progresses in DNA sequencing technologies have enabled efficient genome analysis of crops (Varshney et al. 2014). Combined with suitable genetic resources and good phenotyping protocols, breeders are now capable of implementing crop improvement with a high precision in a short time. We hope that the experiences accumulated in rice improvement would be transferred to other crop species to allow high-speed breeding of crops that can adapt to rapid environmental changes as well as contribute to global food security.

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Chapter 27
Rice Genome Editing

Masaki Endo, Ayako Nishizawa-Yokoi, and Seiichi Toki

Abstract Recently developed methods for genome editing have the potential to accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise or predictable manner. Sequence-specific nucleases (SSNs) induce targeted DNA double-strand breaks (DSBs), and different genome modifications can be achieved depending on the repair pathway. Non-homologous end-joining (NHEJ) repair creates mainly insertions or deletions (in/dels) at the break sites, which can result in frameshift mutations. Such NHEJ-mediated gene modification is called targeted mutagenesis. On the other hand, when a template with homology to the sequence surrounding the DSB is available, DNA DSBs can be repaired by homologous recombination (HR) repair. Such template-mediated HR achieves gene targeting (GT); GT can be used to introduce any desired mutation because the sequence supplied on the repair template is copied and pasted into the endogenous genome. In this chapter, we provide an overview of recent advances in genome-editing technologies in rice.

Keywords Sequence-specific nuclease · CRISPR/Cas9 · Gene targeting · DNA double-strand breaks · Homologous recombination · Non-homologous end-joining

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27.1 Introduction

Precise genome-editing techniques in plants have become an important tool for both the development of plant science and plant molecular breeding. To make the best use of genome-editing techniques in plant molecular breeding, information on the relationship between genes and phenotypes is needed. Rice has long been cultivated as a major crop, and it currently sustains more than half of the world's population. Thus, many different varieties of rice showing different traits are cultivated all over the world. Owing to the abundant plant materials and analysis tools that have been developed in rice—a model monocotyledonous plant—many genes determining plant characteristics have been revealed in rice. So it is no overstatement to say that rice is one of the most preferred plants for genome-editing studies.

Sequence-specific nucleases (SSNs) induce targeted DNA double-strand breaks (DSBs), and different genome modifications can be achieved depending on the repair pathway and the availability of a repair template (Fig. 27.1). The two major DSB repair pathways are non-homologous end-joining (NHEJ) and homologous recombination (HR). In the case of higher plants, NHEJ is dominant, and this error-

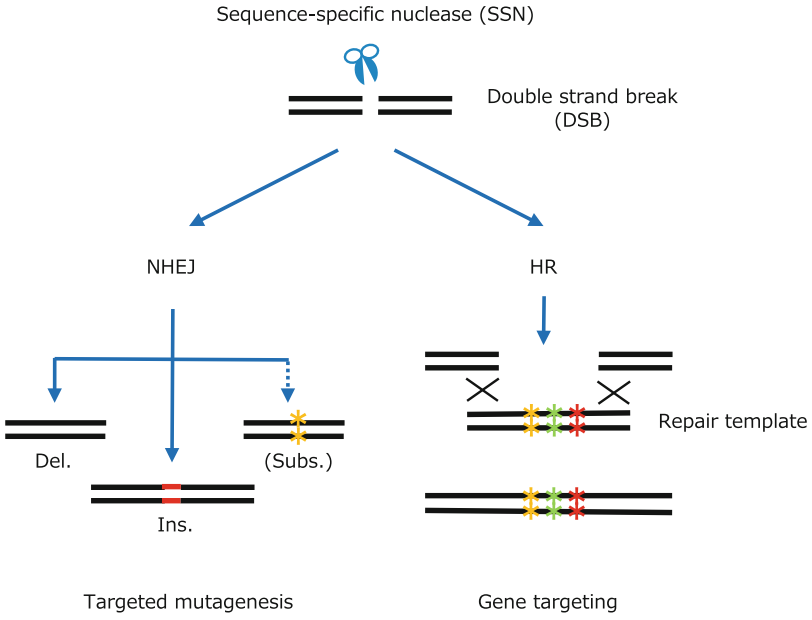

Fig. 27.1 Genome editing with sequence-specific nucleases. DNA DSBs induced by a nuclease at a specific site can be repaired either by non-homologous end-joining (NHEJ) or homologous recombination (HR). Repair by NHEJ usually results in the deletion (Del.) or insertion (Ins.) of random base pairs, causing gene knockout by disruption. Substitutions (Subs.) are rare. When a donor DNA is available, HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or to achieve gene insertion

prone DNA repair pathway makes mainly insertions or deletions (in/dels), which can result in frameshift mutations. However, substitutions rarely occur by NHEJ. This NHEJ-mediated gene modification is called targeted mutagenesis. On the other hand, when a template with homology to the sequence surrounding the DSB is available, DNA DSBs can be repaired by HR, and such template-mediated HR achieves gene targeting (GT). Here, we summarize recent development of genome-editing technologies, mainly targeted mutagenesis and gene targeting in rice.

27.2 Targeted Mutagenesis

Precision targeted mutagenesis has become possible in plants only in the last few years, reflecting the development of reprogrammable SSNs. Early, but limited, success was achieved with modified meganucleases, zinc-finger nucleases (ZFNs). However, the number of successful reports increased rapidly following the development of transcription activator-like effector nucleases (TALENs) (Sprink et al. 2015) and the RNA-mediated SSN CRISPR/Cas9 system, based on the bacterial CRISPR (clustered regularly interspaced short palindromic repeats) system and CRISPR-associated (Cas) nucleases (Cong et al. 2013; Jinek et al. 2012).

TALENs consist of a common nuclease domain derived from FokI, a type IIS restriction enzyme, and distinct DNA-binding domains employing TAL effectors derived from a plant pathogen *Xanthomonas* (Boch et al. 2009; Moscou and Bogdanove 2009). TALENs can be designed to target almost any DNA sequence because each TAL effector module recognizes a single base. Four different modules, each specific to one of the four bases, are used to make TALENs, and these DNA-binding domains can be engineered to target user-defined DNA sequences. Because the FokI nuclease domain must dimerize to cleave DNA (Bitinaite et al. 1998), and the TALEN pair recognizes a 30- to 40-bp DNA sequence, TALENs can realize highly specific targeted mutagenesis. In rice, several examples of successful genome editing using TALENs have been reported (Li et al. 2012; Shan et al. 2015; Zhang et al. 2016; Nishizawa-Yokoi et al. 2016a). Nishizawa-Yokoi et al. (2016a) revealed that a defect in DNA ligase 4 (Lig4) enhanced the frequency of TALEN-mediated targeted mutagenesis and increased the ratio of large deletions (>10 bp).

The advent of the widely used CRISPR/Cas9-derived system has brought genome editing in plants within reach of smaller laboratories equipped with cloning and plant transformation facilities. When targeted mutagenesis is induced by stably integrated DNA encoding Cas9 and single guide RNA (sgRNA), the process for obtaining targeted mutagenized plants is almost the same as standard rice transformation and regeneration. In our system, 1- to 3-week-old calli derived from the scutellum of mature seeds are co-cultivated with *Agrobacterium* harboring the binary vector with Cas9, sgRNA, and selection marker expression constructs (Fig. 27.2). After 3 days, disinfected calli are grown on selection medium containing an appropriate antibiotic. Two to three weeks later, proliferated calli are transferred to regeneration medium, and plants with the targeted mutation are

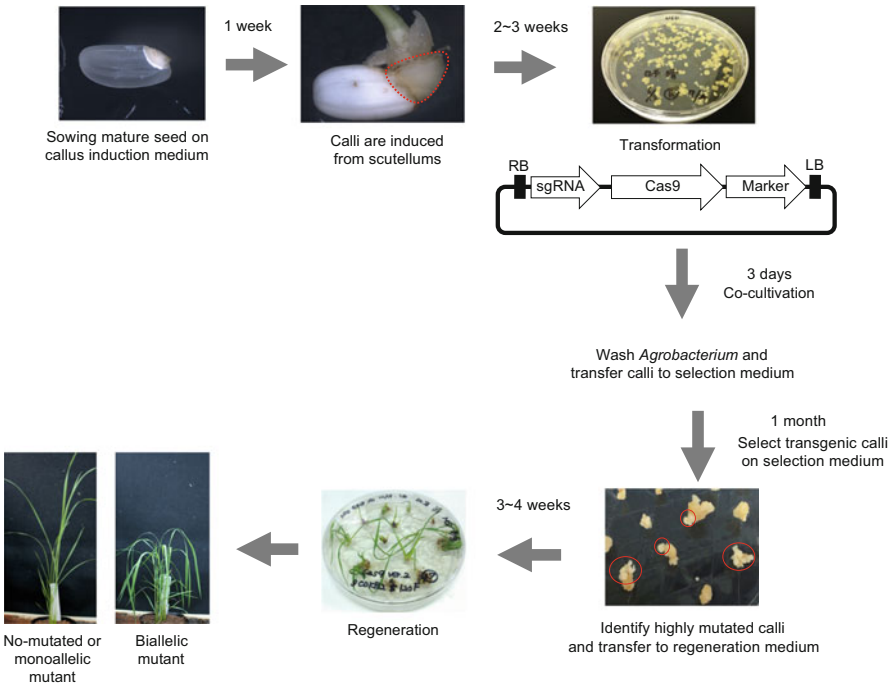

Fig. 27.2 Schematic representation of CRISPR/Cas9-mediated targeted mutagenesis in rice. Calli induced from mature seeds are used for *Agrobacterium*-mediated transformation. Constitutive expression of Cas9 and sgRNA in transgenic cells induces mutations, and regenerated plants originated from mutated cells possess mutations in target gene. Highly mutated calli can be selected by cleaved amplified polymeric sequence (CAPS) analysis when target site of CRISPR/Cas9 is on the restriction enzyme recognition site. Heteroduplex mobility shift assay (HMA) or Guide-it Resolvase assay can be universally used because these assays are based on the detection of mismatches in heteroduplexed DNA of mutated and nonmutated PCR product (for details, see Endo et al. 2016c)

obtained. The chimeric nature of mutated cells and nonmutated cells found in transformed callus can be resolved during the process of regeneration. Mutation frequency differs significantly depending on the Cas9/sgRNA expression construct used and the specific target sequences, and it is relatively easy to obtain regenerated plants with biallelic mutations in the target gene (Mikami et al. 2015). When multi-sgRNAs are expressed, simultaneous targeted mutagenesis at multiple loci and targeted chromosomal deletions can be obtained successfully (Zhou et al. 2014; Lowder et al. 2015).

27.3 Expansion of Selectability and Improvement of Specificity of Targeted Mutagenesis Sites

When *Streptococcus pyogenes* Cas9 (*Sp*Cas9) and sgRNAs are used, CRISPR/Cas9 targets must immediately precede an NGG site protospacer adjacent motif (PAM) sequence recognized by *Sp*Cas9. This requirement for a PAM sequence adjacent to the target site is one of the limitations of this approach. Recently, Cas9 orthologs and other nucleases derived from class II CRISPR–Cas systems have been discovered in bacteria and archaea (Barrangou and Doudna 2016). In the case of rice, successful genome editing using *Sa*Cas9 (Kaya et al. 2016), *As*Cpf1 (Tang et al. 2017), *Lb*Cpf1 (Tang et al. 2017), and *Fn*Cpf1 (Endo et al. 2016a) has been reported. The PAM sequence of *Fn*Cpf1 is TTN, while this sequence is TTTN in *As*Cpf1 and *Lb*Cpf1. Cpf1 differs from Cas9 in at least the following aspects: the PAM is TTN or TTTN, which helps target AT-rich regions and complements the popular *Sp*Cas9 system (NGG PAM); Cpf1 creates 5'-staggered ends, which potentially can facilitate precise gene replacement via NHEJ. For these reasons, CRISPR–Cpf1 is an attractive tool for plant genome editing. Utilization of various Cas9 and Cpf1 that recognize different sequences as PAM expands the selectivity of targeted mutagenesis sites.

CRISPR/Cas9 enables highly efficient genome editing in rice but can also induce mutations at off-target sites that resemble the on-target sequence (Endo et al. 2015). Various strategies have been described to reduce genome-wide off-target mutations of the commonly used *Sp*Cas9 nuclease, including the use of *Sp*Cas9 mutants (Kleinstiver et al. 2015; Slaymaker et al. 2016), or paired *Sp*Cas9 nickases (Mali et al. 2013; Ran et al. 2013) (for details, see Koo et al. 2015). In addition, web-based computer algorithms such as CRISPR-P (Lei et al. 2014, http://cbi.hzau.edu.cn/cgi-bin/CRISPR) and CRISPRdirect (Naito et al. 2015, https://crispr.dbcls.jp), which search for potential off-target sites and unique target sequences in the genome, can help avoid the risk of off-target mutations.

27.4 Base Exchange

Most genome editing induced by CRISPR/Cas9 results in insertions and deletions. While such mutants are very valuable in defining gene function, their application in crop improvement is somewhat limited because many agriculturally important traits are conferred by single-nucleotide polymorphisms or by point mutations. Cas9 has been engineered as a Cas9 nickase with only the D10A mutation (nCas9), or DNA-binding protein without the nuclease activities (dCas9), which has D10A and H840A mutations. dCas9 fused with a cytidine deaminase enzyme was shown to have the ability to convert C to T (or G to A) within a window of approximately 5 nt, providing a very useful tool with which to introduce point mutations at a specific locus defined by a sgRNA molecule (Komor et al. 2016;

Nishida et al. 2016). In rice, Shimatani et al. (2017) used *Petromyzon marinus* cytosine deaminase (*Pm*CDA1) to confer herbicide resistance by inducing a C to T substitution in the acetolactate synthase (*ALS*) gene. Zong et al. (2017) used rat cytidine deaminase APOBEC1 with a uracil glycosylase inhibitor (UGI) that inhibits base-excision repair and succeeded in converting C to T in rice CDC48, NRT1.1B, SPL14, wheat LOX2, and maize CENH3 genes.

27.5 DNA-Free Genome Editing

Genome editing has been achieved by transformation of DNA expressing the nuclease into plant cells, in which the nuclease is either transiently expressed or stably integrated. If integrated, the foreign DNA can be segregated out in the progenies of the mutated plants in case of seed-propagated plants (Fauser et al. 2014).

Delivery of preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs), rather than plasmids that encode these components, into plant cells could remove the likelihood of inserting recombinant DNA into the host genome. Woo et al. (2015) transfected preassembled complexes of purified Cas9 protein and sgRNA into protoplasts of *Arabidopsis*, tobacco, lettuce, and rice and achieved targeted mutagenesis in regenerated plants. However, for most plant species, especially major cereal crops, regeneration of plants from cultured protoplasts, while feasible, is still difficult. Furthermore, there are concerns about somaclonal mutation due to the prolonged tissue culture period. On the other hand, Svitashev et al. (2016) demonstrated that Cas9 and sgRNA in the form of RNP complexes can be delivered into maize embryo cells via particle bombardment; the resulting regenerated plants contained specific targeted mutations. A similar strategy succeeded for wheat genome editing (Liang et al. 2017). Because the mutant plants edited using CRISPR/Cas9 RNPs do not have any integrated transgenes, their application in practical breeding and commercialization should be more publicly acceptable, thus accelerating precision improvement of crops, including rice. Furthermore, as a nucleotide-free genome-editing method, TALENs have been transfected to *Nicotiana tabacum* protoplasts as purified proteins, and TALEN-mediated targeted mutagenesis was successfully observed (Luo et al. 2015).

27.6 Gene Targeting

Gene targeting (GT) allows precise modification of an endogenous target gene, not only for the induction of deletion and insertion mutations but also for introduction of substitution and more complicated mutations through homologous recombination (HR). In rice, a highly reproducible GT procedure has been established and has been utilized to generate both loss-of-function and gain-of-function mutants (Terada et al. 2002, 2007; Endo et al. 2007; Saika et al. 2011; Nishizawa-Yokoi

et al. 2015a, 2016b). The development of an efficient selection system for GT cells was essential for the establishment of a reproducible GT procedure in rice, since the ratio of cells carrying the target gene modified via GT to all transgenic cells is thought to be quite low (Puchta 2002; Hanin and Paszkowski 2003).

27.7 GT with Gene-Specific Selection

One of the successful applications of targeted gene modification via GT has made use of gene-specific selection, in which the mutation introduced into the target gene via GT confers a selectable trait, such as herbicide resistance (Fig. 27.3a). Ten years ago, our group found that the introduction of two point mutations (leading to two amino acid changes) into the rice acetolactate synthase (*ALS*) gene via GT conferred herbicide bispyribac sodium (BS) tolerance in rice (Endo et al. 2007). Subsequently, we successfully produced, by GT, tryptophan-accumulating rice plants with introduced point mutations conferring resistance to feedback inhibition by tryptophan or its analogs and enhancing the catalytic activity of the α-subunit of anthranilate synthase—a key enzyme in the tryptophan biosynthetic pathway (Saika et al. 2011). As shown in Fig. 27.3a, a GT vector containing a 7- to 8-kb fragment of the target gene locus with a truncated target gene lacking its promoter and N-terminal region, and carrying the desired mutations, was transfected into rice calli. Transgenic calli were selected directly on medium containing the herbicide BS or the tryptophan analog 5-methyl-tryptophan, i.e., allowing gene-specific selection. Although almost all GT vectors introduced into cells integrated randomly into any genomic locus via the NHEJ pathway, transgenic cells with random integration of the GT vector do not exhibit any resistance to the herbicide or tryptophan analog. Conversely, GT calli in which GT vector has integrated into the target locus by HR can survive and proliferate on the selection medium. Thus, gene-specific selection is a convenient and effective approach for the identification of true GT cells and has been applied successfully to *Arabidopsis* (Hanin et al. 2001; Endo et al. 2006). However, this approach can be applied only in cases where introduction of the mutation into the target gene confers a selectable trait.

27.8 GT with Positive–Negative Selection

A positive–negative selection system developed in mammalian cells (Mansour et al. 1988) has been applied to the identification of GT cells from among a large number of transgenic cells in rice (Terada et al. 2002). This approach aims to eliminate transgenic cells harboring randomly integrated T-DNA and enrich GT cells by employing a GT vector carrying both a positive and a negative selection marker (Fig. 27.3b). In rice, GT using the hygromycin phosphotransferase (*hpt*) gene as a positive selection marker and the diphtheria toxin A-fragment (*DT-A*)

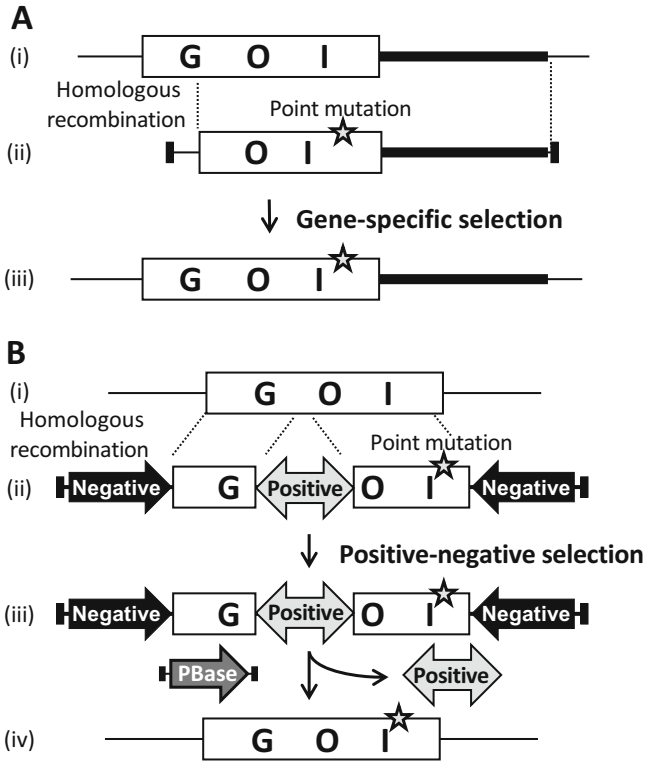

Fig. 27.3 Strategy for targeted gene modification via GT in rice. (**a**) Strategy for introduction of point mutations (a star) via GT with gene-specific selection. This approach can be applied to gene modifications that confer a selectable trait such as resistance to herbicides. (**b**) Strategy for precise gene modification via GT with positive–negative selection and subsequent marker excision. Using *piggyBac* transposon for marker excision without leaving any dispensable sequences, we can produce the mutant plants harboring only desired mutations in target gene. This approach is available for modification of any gene of interest. GOI, gene of interest. (*i*), wild-type GOI locus; (*ii*), GT vector; (*iii*), modified GOI locus via GT; (*iv*), modified GOI locus carrying only point mutation resulting from GT and subsequent marker excision

gene as a negative selection marker has become a universal approach for modifying any target gene; successful examples now include *Waxy* (Terada et al. 2002), alcohol dehydrogenase 2 (*Adh2*, Terada et al. 2007), methyltransferase 1a (*MET1a*, Yamauchi et al. 2009), *MET1b* (Yamauchi et al. 2014), domains rearranged methylase 2 (*DRM2*, Moritoh et al. 2012), Repressor of Silencing 1 (*ROS1*, Ono et al. 2012), *Rac1* (Dang et al. 2013), and Flowering locus T (*FT*, Tamaki et al. 2015). Using positive–negative selection, an expression cassette with the positive selection marker gene is retained within the modified target locus via GT. To leave only the desired mutation in the target gene, the positive selection marker gene needs to be excised completely from the target gene. Site-specific recombination systems such as Cre/*loxP* and transposons such as *Ac*/*Ds* have been

used widely to remove selectable marker genes from host plant genomes (reviewed in Hare and Chua 2002; Woo et al. 2011). Site-specific recombination systems have also been utilized to remove selectable marker genes from GT loci in rice (Terada et al. 2010; Dang et al. 2013). A major problem in marker excision systems using site-specific recombinase and a transposon is the presence of residual sequences, e.g., the recognition sequences for the site-specific recombinase, and a footprint, at the excised site. To address this issue, we developed a precise marker excision system that leaves no dispensable sequences behind. We focused on the *piggyBac* transposon derived from the lepidopteran cabbage looper moth *Trichoplusia ni* (Cary et al. 1989) because of its well-known ability to excise without leaving a footprint at the excised site in animal systems (Yusa 2015). We revealed for the first time that the animal-derived *piggyBac* can transpose efficiently and accurately in plants (Nishizawa-Yokoi et al. 2015b) and have applied *piggyBac* successfully to marker excision from a GT locus (Nishizawa-Yokoi et al. 2016b).

Rice calli were transfected with *Agrobacterium* harboring a GT vector carrying the *hpt* gene as a positive selection marker within *piggyBac* transposon and the *DT-A* gene as a negative selection marker (Fig. 27.3b). About 1% of hygromycin-resistant calli were identified as GT calli using PCR analysis, and GT calli were transfected with *Agrobacterium* harboring a *piggyBac* transposase (PBase) expression cassette to excise the positive selection marker gene. More than 90% of regenerated plants expressing PBase were found to be marker-free rice plants, with no reintegration of *piggyBac* into other loci. Moreover, we obtained T_1 progeny plants with only the desired mutations via GT in the target gene and with no *hpt* or *PBase* gene present (Fig. 27.3b). This approach therefore allows precise modification of any gene of interest via GT in rice.

Although the described GT procedure with positive–negative selection using the *DT-A* gene has been utilized widely for modification of target genes in rice, there have been no reports of adaptation of this approach to other plant species. One of the reasons why GT with positive–negative selection using the *DT-A* gene as a negative selection marker is not suitable for other plants is that GT cells might be lost by transient expression of the *DT-A* gene from the GT vector prior to T-DNA integration into the plant genome. Thus, we attempted to establish a conditional negative selection marker gene, in which the negative selectable effect can be regulated by treatment of the transgenic cells with a specific agent. Cytosine deaminase (*codA*) derived from *Escherichia coli* converts nontoxic 5-fluorocytosine into the toxic 5-fluorouracil (Perera et al. 1993), making *codA* a useful conditional negative selection marker in several plant species (Serino and Maliga 1997; Koprek et al. 1999; Shao et al. 2015; de Oliveira et al. 2015). Studies in our laboratory have shown that a mutant *codA* carrying a single amino acid change (codA D314A) could be used as a negative selection marker to enrich GT cells in rice (Osakabe et al. 2014). More recently, we established a novel positive–negative selection system consisting of a combination of *neomycin phosphotransferase II* (*nptII*) and an antisense *nptII* construct: the *nptII-antinptII* system (Nishizawa-Yokoi et al. 2015a). Our findings indicated successful use of the *nptII-antinptII* system to modify endogenous genes via GT in rice, although the

negative selection pressure of anti *nptII* was lower than that of *DT-A*. Nevertheless, this approach is expected to be applied to many plant species because *nptII* is the marker gene most widely available in plants.

27.9 Attempts to Improve GT Frequency

Even in rice using positive–negative selection, the efficiency of isolation of GT cells has been shown to be low. Thus, we have attempted to establish a high-efficiency GT procedure using several approaches. In one such approach, we reported that suppression of the NHEJ-related genes, *Ku70*, *Ku80*, and DNA ligase 4 (*Lig4*), led to a decreased frequency of T-DNA integration and enhanced activity of the HR pathway in rice (Nishizawa-Yokoi et al. 2012). Moreover, our studies demonstrated that overexpression of rice *RecQl4* (Bloom helicase counterpart protein) and/or exonuclease1, which play a role in the process of the resection of 5′ ends of the DSBs in the first step of HR, could enhance HR and/or single-strand annealing (SSA) repair as evaluated by a reporter construct in rice (Kwon et al. 2012). Suppression of the NHEJ pathway and/or overexpression of HR-related genes is expected to cause an increase in the occurrence of HR-mediated GT and suppression of T-DNA random integration by NHEJ, resulting in a synergistic effect that will improve GT frequency.

On the other hand, the induction of DSBs at target genes via SSNs has allowed the development of an efficient GT approach in plants. Qi et al. (2013) showed enhanced GT efficiency by ZFNs-induced DSBs in *Arabidopsis* protoplasts. They also reported that ZFN-mediated GT was greatly enhanced by loss of *Ku70*, *Lig4*, and *SMC6b* involving sister chromatid alignment (Qi et al. 2013). Li et al. (2013) revealed that CRISPR/Cas9-mediated GT occurred in more than 10% of *Nicotiana benthamiana* protoplasts. In mammalian cells, it has been reported that the suppression of NHEJ-related genes by gene silencing and treatment with *SCR7*, a *Lig4* inhibitor, promoted the efficiency of HR-mediated GT using CRISPR/Cas9 (Chu et al. 2015; Maruyama et al. 2015). Treatment with the chemical compound Rad51-stimulatory compound 1 (RS-1), known as an activator of Rad51 DNA-binding activity (Jayathilaka et al. 2008), also improved SSN-mediated GT efficiency in rabbit embryos (Song et al. 2016). In treatment of human ES/iPS cells, the valproic acid, a histone deacetylase inhibitor, increased the efficiency of CRISPR/Cas9-induced biallelic GT efficiency (Takayama et al. 2017). In the case of SSN-mediated GT in plant cells, double-stranded DNA fragments or plasmid DNA have been utilized as a GT donor (Qi et al. 2013; Li et al. 2013), while SSN-mediated GT using single-stranded oligodeoxynucleotides (ssODNs) as a GT donor has been employed to introduce single amino acid substitutions and small deletions/insertions in a wide range of animal species, including mouse (Shen et al. 2013), human (Rivera-Torres et al. 2017), chicken (Wang et al. 2017a), pig

(Wang et al. 2016), and zebra fish (Armstrong et al. 2016). Yoshimi et al. (2016) demonstrated the use of a 1-kb-long ssODN as a GT donor for targeted insertion of long fragments (500 to 800-bp) via HR into a CRISPR/Cas9 targeting site in rat fertilized eggs.

In addition, a novel GT method, called *in planta* GT, has been developed in *Arabidopsis* by Fauser et al. (2012). In this system, a GT donor vector carrying two recognition sites for SSNs at both ends was integrated into the genome prior to SSN expression. Following subsequent SSN expression, DSBs were induced within the target gene and at both ends of GT donor in the genome, and the GT donor was spliced out and supplied to direct DSB repair of the target gene by HR. We have successfully applied this system to rice (Endo et al. 2016b). Although there was no significant difference in GT frequency between transgenic cells carrying CRISPR/Cas9-mediated DSB induction at the target locus and GT donor in the genome and control cells carrying no expression of Cas9, we found that in planta GT efficiency was markedly stimulated by concomitant knockout of *Lig4* via CRISPR/Cas9. Surprisingly, one transgenic line exhibiting biallelic GT at the target locus was obtained using this approach (Endo et al. 2016b).

An efficient approach to deliver the SSN expression cassettes and GT donor vectors is to use a geminivirus vector. Geminivirus vectors replicate via extrachromosomal rolling-circle replication in the host cell nucleus, making precise modification via HR-mediated GT possible in tobacco (Baltes et al. 2014), tomato (Cermak et al. 2015), potato (Butler et al. 2016), and wheat (Gil-Humanes et al. 2017). These reports demonstrate that a high level of transient expression of SSNs, and abundance of the GT donor in host nucleus, can be achieved using a geminivirus vector. In addition, geminivirus and wheat dwarf virus, which can replicate in rice cells (Laufs et al. 1990), were applied to a system delivering SSN expression cassettes and GT donors in rice (Wang et al. 2017b).

27.10 SSN-Mediated Homology-Independent Knock-in System

As described above, HR-mediated GT is inefficient in most higher organisms, because HR activity, which depends on cell cycle stage, is nearly absent in G1/M and is high in S and G2 phases of the cell cycle. By contrast, NHEJ is a dominant pathway throughout the cell cycle (Yun and Hiom 2009; Chapman et al. 2012). Thus, HR-mediated GT can occur only in actively dividing cells, including tissue culture cells. Recently, NHEJ-mediated targeted transgene integration systems, which allow targeted gene modification in nondividing cells, including somatic cells, have been developed successfully in animal cells (Nakade et al. 2014; Aida et al. 2016; Suzuki et al. 2016). Nakade et al. (2014) reported microhomology-

mediated end-joining (MMEJ)-dependent targeted integration of donor DNA, termed the PITCh (Precise Integration into Target Chromosome) system, which enabled efficient knock-in of donor DNA into a target locus by induction of DSBs via SSNs in both the target gene and the donor DNA and subsequent integration of donor DNA via MMEJ. PITCh systems have been applied to gene modification via knock-in in human cells, silkworms, frogs (Nakade et al. 2014), and zebra fish (Kawahara et al. 2016). Furthermore, the efficiency of the PITCh system had been enhanced successfully by the use of 40-bp microhomology between the target gene and donor DNA and expression of Exo1—an MMEJ-related gene (Aida et al. 2016). Suzuki et al. (2016) established a homology-independent targeted integration (HITI) strategy—an NHEJ-mediated targeted transgene integration system that is more efficient compared with HR-mediated GT and the PITCh system in mammal cells. Surprisingly, Suzuki et al. (2016) reported that an HITI strategy allowed for knock-in of donor DNA into the target locus in nondividing cells of adult mouse tissues, including the brain and retina.

To date, there have been no reports on SSN-mediated homology-independent knock-in systems, such as PITCh and HITI, in plants. By applying such approaches in plants, it might be possible to establish such knock-in strategies in agriculturally important crops, even if the frequencies of transformation are quite low.

27.11 Conclusions

As explained in this chapter, multiple new tools have been developed for plant genome editing. Once a link between a particular nucleotide variation and a desired phenotype/trait has been verified, the same variation can be introduced into other varieties or elite lines. Direct modification of the genomes of rice varieties of interest/elite lines through genome-editing techniques can result in the introduction or restoration of desired traits and reduces the time required for introgression of the allele by avoiding backcrossing of a large number of inbred lines. We hope that genome editing technology can contribute to solving both environmental issues and problems of food security.

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Chapter 28
Databases for Rice Omics Studies

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Abstract In modern molecular biology, databases play a pivotal role. With the advent of high-throughput DNA sequencing technologies, a central question has become how to process, store, and present large-scale data sets, and databases where researchers can search for sequence data and related omics information are now indispensable. For rice, two major genome databases, the RAP-DB and RGAP databases, and several transcriptome databases are widely used. As genome resequencing data increased, some databases capable of displaying multiple genomes were also developed. Furthermore, novel databases are being developed for the comparison of genome sequences among wild and cultivated *Oryza* species. Bioinformatics analyses of omics information will be needed in the future, and researchers will likely desire to effectively retrieve data created by such analyses; therefore, databases are expected to function as a hub for multiple rice omics resources. Consequently, databases will facilitate the next-generation breeding science based on large-scale omics data.

Keywords Database · Bioinformatics · High-throughput DNA sequencing · Genome · Transcriptome · Comparative omics

28.1 Biological Databases in the Era of Omics

Several areas of biology, which aim to analyze biological entities or phenomena as a whole, are often referred to as "omics." The concept of omics has become useful since massive data productions were accelerated in a number of areas of biology through technological innovations. For example, the limited DNA sequencing capacity of twentieth-century technologies once constrained molecular biological studies such that only gene-by-gene approaches were possible. Later, innovative

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advancements of DNA sequencing methods revolutionized molecular biology (Goodwin et al. 2016). Now, genomics, which addresses a whole set of genes, has been made easy even in a small-scale personal research project. In this way, since omics studies are realized using massive data productions, they naturally coincide with bioinformatics for processing a tremendous amount of biological data. To utilize such data, one can easily deduce that high-performance computation should play a pivotal role because the growth of biological data is even faster than the prediction of Moore's law (Muir et al. 2016). In particular, one of the central subjects of modern molecular biology is how efficiently we can retrieve useful information from a bulk of data. Herein lies the problem underlying the urgent need for molecular biological databases. The database is not merely a collection of data in a computer system, but a requirement is that the contents can be searched easily so that users can find information in a timely manner. For this reason, popular molecular biology databases are usually equipped with the following functionality. First, the data are related to other useful information and the linkages between the data are visible. For example, sequence information linked to genetic resources should expedite agricultural application of genome-wide studies (Kurata and Yamazaki 2006). Second, the search functions are intuitive. While keyword search is widely used, DNA or protein sequence search is equally useful or even more anticipated in molecular biology. Third, downloading bulky data is possible. This functionality is particularly useful for bioinformaticians who can conduct data analyses on their own. In addition to these features, openness is also an essential feature. Most of the users expect that online data are available at any time free of charge. Taken together, data accessibility and flexibility are required for biological databases. Although a large number of databases have been released to the public in the past, a limited number of them have such functionality. In this chapter, we select and present such useful information resources for the readers' convenience.

28.2 Genome Databases of *O. sativa* (*japonica*)

Genome databases provide genomic sequences as well as various types of related data, including structural, functional, and experimental information of genes. In particular, the primary databases generally harbor the latest version of the original (complete) genome assembly of a specific species together with its annotation data. The secondary databases that utilize such genome information present some analyzed data and sometimes include additional experimental results, including other omics data. For the *japonica* cultivar of *Oryza sativa*, there are two major primary databases: one is the Rice Annotation Project Database (RAP-DB) of National Agriculture and Food Research Organization (Itoh et al. 2007; Sakai et al. 2013) and the other is the Rice Genome Annotation Project Database of Michigan State University (MSU) (Yuan et al. 2003).

To provide accurate rice genome annotation data on the high-quality *japonica* rice genome sequence that was deciphered by the International Rice Genome Sequencing Project (IRGSP), an international collaboration, the Rice Annotation Project (RAP), was launched in 2004, and the RAP-DB (http://rapdb.dna.affrc.go.jp/) was later released as its outcome (Ohyanagi et al. 2006). RAP annotation has been the sole official data of the IRGSP genome sequence from its Build 3 release, which was published in 2005 (Ohyanagi et al. 2006). Since the importance of the manual correction of annotation data was stressed in the early stage of the genome sequencing era (Kyrpides and Ouzounis 1999), RAP aimed to thoroughly examine all the descriptions of the functions of rice genes. To achieve this goal, a series of jamboree-style annotation conferences were organized, and comprehensive manual curation based on the literature was conducted. While genome sequences are usually published with various annotated data, including the primary gene structures, gene functions, and repeats, these data are often determined by automated gene prediction only; thus, the curation, involving manual examination of biological data by experts, was required for various species (Kyrpides and Ouzounis 1999; Devos and Valencia 2001; Schnoes et al. 2009). In the case of RAP, we referred to the H-Invitational Project model (Imanishi et al. 2004), where the gene descriptions were manually curated in an annotation jamboree. Additionally, because the number of rice genes had been inflated by erroneous computational predictions (Bennetzen et al. 2004; Cruveiller et al. 2004), we decided to conduct transcript-based annotation of the gene structures so that virtually all the exon-intron structures would be based on experimental evidence, such as full-length cDNAs (Itoh et al. 2007). Regarding the gene identifiers, RAP follows the rice system of the Committee on Gene Symbolization, Nomenclature and Linkage (CGSNL). The gene symbols defined by the CGSNL (McCouch and CGSNL 2008) were fully applied to the gene annotation in the RAP-DB.

The RAP-DB has the following four basic functions (Fig. 28.1): (1) genome browser, (2) search functions, (3) data download, and (4) other functions, which are usually equipped with popular genome databases. The genome browser is used for the visualization of genomic sequences as well as various types of annotation data. In the RAP-DB, there are 126 types of data categorized into 17 large groups, which include the RAP data, RNA-Seq data, and other annotations. The search functions are for the keywords in the text and biological sequences. For the text search, users can query identifiers, gene symbols, DNA marker names, functional descriptions, and any other keywords. The BLAST (Altschul et al. 1997) and BLAT (Kent 2002) sequence searches enable users to discover the genomic regions or genes by querying nucleotide or amino acid sequences of interest. For those who desire to conduct massive data analysis, the genome sequence, its annotation, and other related data are available directly from the download page. Additionally, RAP-DB functions in converting identifiers between two different genome annotations, RAP and MSU. Furthermore, to generate synergy between the genome and other data, the gene annotation data of RAP-DB is hyperlinked to other databases: DDBJ (http://www.ddbj.nig.ac.jp/), NCBI (https://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/), Oryzabase (https://shigen.nig.ac.jp/rice/

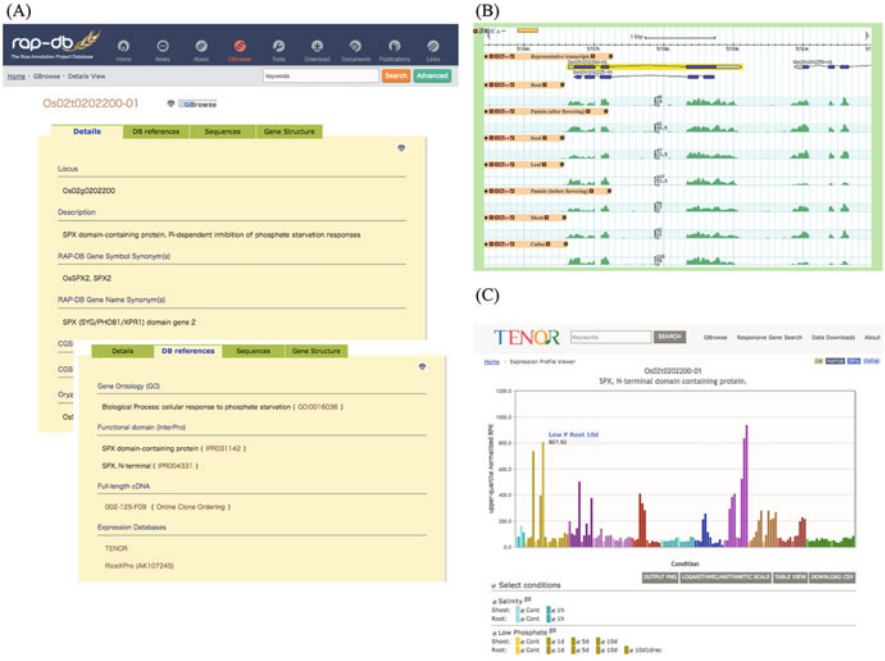

Fig. 28.1 The Rice Annotation Project Database (RAP-DB) and Transcriptome ENcyclopedia of Rice (TENOR). (**a**) Gene annotation in the RAP-DB. The links to the genome browser and TENOR are shown. (**b**) The distributions of the RNA-Seq reads are presented to display the transcriptional activities at a 1 bp resolution. (**c**) The expression profile view of TENOR shows the normalized expression levels under 12 growing conditions

oryzabase/), Gene Ontology (http://amigo.geneontology.org/amigo/landing), InterPro (http://www.ebi.ac.uk/interpro/), DNA Bank (http://www.dna.affrc.go.jp/distribution/), TENOR (http://tenor.dna.affrc.go.jp/), and KEGG (http://www.genome.jp/kegg/). The contents of RAP-DB are still actively maintained, and curated descriptions of gene functions and gene structures are released several times a year.

The Rice Genome Annotation Project (RGAP) database (http://rice.plantbiology.msu.edu/) was developed by a group of MSU (Fig. 28.2). This database, which was formerly known as the "osa1" database of The Institute for Genomic Research (TIGR), constructed another rice genome assembly that differed from that of IRGSP and presented an independent annotation resource (Yuan et al. 2003; Yuan et al. 2005), though TIGR was also a part of IRGSP. Later, for the users' convenience, the rice genome assemblies were unified into one as IRGSP-1.0 by RAP and MSU (Kawahara et al. 2013), and both annotation data were created on the same rice reference genome. As is the case for the RAP-DB, the RGAP database

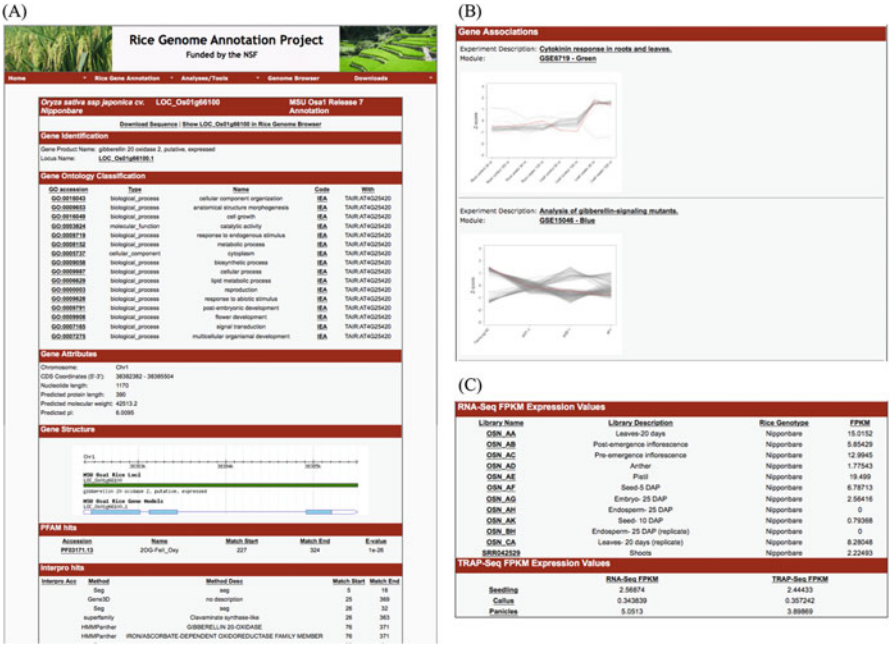

Fig. 28.2 MSU's Rice Genome Annotation Project (RGAP) database. (**a**) Gene annotation in the RGAP database. (**b**) Trend plots of the microarray expression profiles. (**c**) Expression levels (FPKM values) of the RNA-Seq and TRAP-Seq experiments

provides various functions, such as a genome browser and search functions. Since this group annotated not only rice but also other species, the database can be used for comparative genomics of rice and these related species. The latest annotation of RGAP is Release 7 in 2013 (Kawahara et al. 2013).

Recently, using the IRGSP-1.0 genome sequence registered in the International Nucleotide Sequence Database Collaboration (INSDC) (Blaxter et al. 2016), the National Center for Biotechnology Information (NCBI) has started to release its own rice genome annotation (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Oryza_sativa_Japonica_Group/101/). The annotation was created by an original annotation system, NCBI Eukaryotic Genome Annotation Pipeline. Among the three independent annotations, the numbers of the annotated genes vary: 37,852 in the RAP-DB, 39,045 in RGAP, and 30,724 in NCBI. The RAP-DB and RGAP have some additional genes, with 8117 computationally predicted genes in the RAP-DB and 16,941 transposon-related genes in RGAP. This difference in the annotation data should be due to the different computational systems employed in the RAP-DB and RGAP. For instance, the RAP-DB genes were demonstrated by full-length cDNAs, while the RGAP genes were based on massive parallel signature sequencing data (Nakano et al. 2006). It is anticipated that these annotations will be updated by RNA-Seq or any other data generated by state-of-the-art technology.

28.3 Genome Databases of *O. sativa* (*indica*)

As was previously mentioned, the *japonica* cultivar databases have long been maintained and have rich contents. In contrast, the development of genome databases for the *indica* cultivars is insufficient. Shortly after the publication of an *indica* cultivar, 93-11, in 2002, BGI-RIS (http://rise2.genomics.org.cn/page/rice/index.jsp) was developed to present the genome information of *indica* (Yu et al. 2002; Zhao et al. 2004). Later, this database was updated (He and Wang 2007) so that comparisons of three different types of rice genomes, 93-11 (*indica*), Nipponbare (*japonica*), and PA64s (created by multiple crossing of *indica*, *temperate japonica*, and *tropical japonica*), could be visualized. Unfortunately, this database employed an obsolete draft genome of *japonica* (Goff et al. 2002), and it was not updated after 2010.

The *indica* group is known to encompass a wide variety of cultivars. In particular, the *aus* group is often regarded as an independent group that differs from the standard *indica* varieties (Garris et al. 2005), and hence it is of great value to have the genome of *aus* determined and to make a comparison among the wide variety of cultivars. In 2014, the genome of Kasalath, a cultivar of *aus*, was released (Sakai et al. 2014); later, the genomes of IR64 (*indica*) and DJ123 (*aus*) followed (Schatz et al. 2014), although these genome assemblies are extensively fragmented. Currently, no high-quality genome assembly comparable with the *japonica* reference genome is available for *indica*; this is part of the reason for the quantitative and qualitative deficiency of the *indica* rice genome databases.

28.4 Transcriptome Databases

The genomic sequences are useful for obtaining a static view on the genetic basis, on the one hand. On the other hand, the transcriptomic data enable us to understand the dynamic processes of living organisms. Thus far, a number of large-scale gene expression analyses of both oligonucleotide microarrays and massively parallel sequencing technologies have been reported. In particular, RNA-Seq, which is currently more prevalent than microarray-based methods, allows us to perform comprehensive transcriptome analyses in a fast and cost-effective manner (Wang et al. 2009). In addition to the expression information, novel primary structures of transcripts can be defined if RNA-Seq reads are aligned to the reference genome. This section provides information about expression profiles and co-expression networks from transcriptome databases under various conditions (Table 28.1).

The two representative genome annotation databases, the RAP-DB and MSU's RGAP database, also contain publicly available gene expression data produced by the microarray and RNA-Seq technologies. In the RGAP database, the gene expression data, as well as the functional annotation (gene functions, functional domains, homologous proteins, orthologous genes, etc.), are described for each locus. The

Table 28.1 Web services for transcriptome data retrieval and analysis

Database name	URL
ATTED-II	http://atted.jp
Field Transcriptome Database (FiT-DB)	http://fitdb.dna.affrc.go.jp
OryzaExpress	http://plantomics.mind.meiji.ac.jp/OryzaExpress/
Plant Omics Data Center (PODC)	http://bioinf.mind.meiji.ac.jp/podc/
RiceArrayNet	http://bioinfo.mju.ac.kr/arraynet/
Rice Expression Database	http://expression.ic4r.org
RiceFREND	http://ricefrend.dna.affrc.go.jp
RicePLEXdb	http://www.plexdb.org/plex.php?database=Rice
RiceXPro	http://ricexpro.dna.affrc.go.jp
Transcriptome ENcyclopedia of Rice (TENOR)	http://tenor.dna.affrc.go.jp

expression levels based on some RNA-Seq experiments and the trend plots based on the microarray expression profiles under various conditions and tissues are also shown (Fig. 28.2). In the RAP-DB, the distribution of the aligned RNA-Seq reads from the seven tissues (shoot, root, panicle, callus, etc.) and some abiotic stress conditions (high salinity, low and high phosphate, etc.) are shown in the genomic feature tracks (Fig. 28.1). TENOR, one of the satellite databases of the RAP-DB, is specialized to display rice gene expression profiles based on RNA-Seq experiments under ten abiotic stress conditions (high salinity; high and low phosphate; high, low, and extremely low cadmium; drought; osmotic pressure; coldness and flood) and two plant hormone treatment conditions (abscisic acid and jasmonic acid) (Fig. 28.1) (Kawahara et al. 2015). Furthermore, data from novel unannotated genes predicted by means of the alignment of RNA-Seq data, co-expressed genes, and cis-regulatory elements within the promoter region of each transcript are retrievable. The expression profiles stored in TENOR are mutually linked to the gene annotation descriptions in RAP-DB so that the users can refer to both time-course transcriptome data and gene functions.

Recently, the Rice Expression Database (RED), another integrated database of rice gene expression profiles derived from publicly available RNA-Seq data, was published (Xia et al. 2017). RED integrates a large number of gene expression profiles (284 RNA-Seq experiments obtained from the databases of the INSDC) that encompass a broad range of rice growth stages and cover a wide variety of biotic and abiotic treatments (Fig. 28.3). RED can display expression profiles and co-expression networks through web-based graphical user interfaces.

Some large-scale transcriptome databases covering multiple plant species, such as rice, maize, wheat, and *Arabidopsis*, are currently available. The Plant Expression Database (PLEXdb) provides a unified gene expression resource based on the microarray technique for both plants and their pathogens (Dash et al. 2012). For instance, gene expression profiles of rice and rice blast fungus are available in RicePLEX and PathoPLEX, respectively. The expression profiles in various

Fig. 28.3 Rice Expression Database (RED). Expression profiles based on publicly available RNA-Seq data are shown using a: (**a**) line chart, (**b**) heatmap, and (**c**) boxplot. (**d**) Co-expression network. (**e**) Distributions of RNA-Seq reads

developmental stages can be shown for each probe of the Affymetrix 57k Rice GeneChip and 20k NSF rice long oligonucleotide array; RicePLEX also provides web-based analytical tools to draw a scatterplot of the expression levels of all probes and to compare the expression levels between two experimental conditions.

Comprehensive gene expression profiles obtained from various tissues or organs, from different developmental stages and under biotic and abiotic stress conditions, are of great use to infer the molecular functions of genes. In particular, the similarity in the gene expression (co-expression gene network information) across a wide range of conditions can be used for the efficient characterization of gene functions and the inference of signaling networks. For rice, co-expression gene network information is available from RED, RiceArrayNet, OryzaExpress, ATTED-II, and RiceFREND (Lee et al. 2009; Hamada et al. 2011; Sato et al. 2013b; Aoki et al. 2016). PlantExpress is a platform for gene expression network (GEN) analysis within a single species or between different species (Kudo et al. 2017). Several rice gene expression data sets generated by a Rice Gene Expression

Microarray 4×44K (Agilent) and GeneChip Rice Genome Array (Affymetrix) are presented in OryzaExpress, which is a sub-platform of PlantExpress for a GEN analysis in rice. The Plant Omics Data Center (PODC) was developed to provide a next-generation sequencing-derived GEN, functional annotations, and additional comprehensive omics resources of multiple plant species, including rice (Ohyanagi et al. 2015).

In the past, it was an accepted standard to conduct experiments under a controlled laboratory condition so that a scientific comparison of the data would be easy. Most of the publicly available transcriptome data were collected in a laboratory. However, in the era of large-scale biology, researchers started to envision gene expression measurements in the field, and several research groups performed field studies of the rice transcriptome. RiceXPro provides a time series of gene expression profiles of mature leaves during a time span from transplant until harvest in a paddy field in Tsukuba, Japan (Fig. 28.4) (Sato et al. 2013b). Various types of gene expression data, such as spatiotemporal gene expression profiling based on 48 different tissues and organs at various developmental stages, are also available in the latest version of RiceXPro (Sato et al. 2013a). Referring to the field transcriptome data in RiceXPro, Nagano et al. developed a statistical modeling method to predict genome-wide mRNA expression profiles, which uses the endogenous diurnal rhythms and variable environmental data (air temperature, solar radiation, humidity, etc.) of rice (Nagano et al. 2012). The outcomes of this statistical modeling method of transcriptomic dynamics in the field condition predict gene expression profiles and parameters of the best-fit model for each gene and are all available in the Field Transcriptome Database.

28.5 Databases for Comparative Omics

As the omics information increases for multiple species, integrated databases in which data can be compared between species are of great use. For such comparative studies, Gramene, Ensembl Plants, MIPS PlantsDB, and PlantGDB are popular databases (Ware et al. 2002; Dong et al. 2004; Kersey et al. 2010; Nussbaumer et al. 2013). In particular, Ensembl Plants (http://plants.ensembl.org/) is maintained by the European Bioinformatics Institute in collaboration with the Gramene database (http://www.gramene.org/) of Cold Spring Harbor Laboratory so that the information resources and technologies can be shared between these two databases. They provide omics data from *japonica* and *indica* rice as well as data obtained from other *Oryza* species and other plants, such as wheat, barley, maize, soybean, and *Arabidopsis*.

The INSDC members, DDBJ of Japan, EMBL-EBI of Europe, and NCBI of the USA, host a data repository for large-scale sequencing data: DDBJ Sequence Read Archive (DRA, http://trace.ddbj.nig.ac.jp/dra/index.html), European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena), and Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra). For rice, we tried the following keywords:

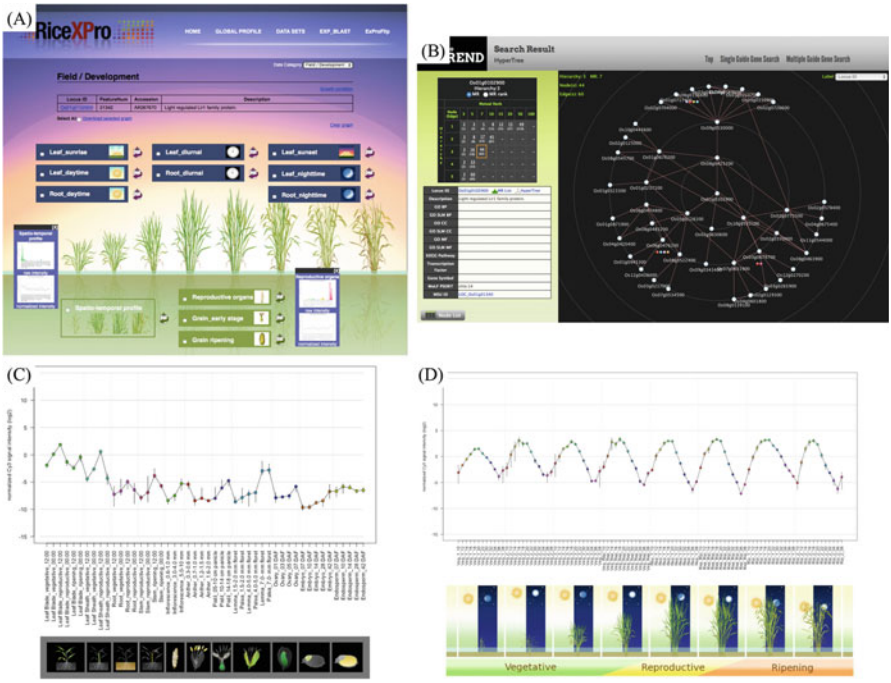

Fig. 28.4 RiceXPro and RiceFREND. (**a**) Gene expression profiles and (**b**) the co-expression gene network under various conditions are displayed for each locus. (**c**) Normalized expression profiles of the various organs and tissues. (**d**) Normalized expression profiles during the growth of rice in the field

"Oryza sativa" in [Organism] and genome in [All Fields] at NCBI's SRA. As a result, 35,218 items were reported as of August 23, 2017. Therefore, provision of a database for the comparison of rice cultivars is an urgent issue. For this purpose, the International Rice Research Institute (IRRI) has developed the Rice SNP-Seek Database (http://snp-seek.irri.org/) (Alexandrov et al. 2015), which harbors 3000 rice genomes, as well as their phenotype and variety information (The 3000 rice genomes project 2014). This is a part of the international collaboration of IRRI, the Chinese Academy of Agricultural Sciences, and Beijing Genomics Institute, in which 10,000 rice core germplasms are to be re-sequenced and whole genome molecular design breeding is to be conducted as a part of the Green Super Rice Project. In addition to such a big genome resequencing project, individual research groups have been generating an enormous amount of sequence information of rice cultivars and wild accessions. To cope with the deluge of sequence data, a web browser, TASUKE (http://tasuke.dna.affrc.go.jp/), was developed to display hundreds of genomes in an effective manner (Kumagai et al. 2013). TASUKE can be used as a personal genome sequence viewer, and it is useful to release genome resequencing data through the Web. PopGeV (http://www.soyomics.com/popgev/)

is a similar web-based application to visualize a massive sequence data set (Shi et al. 2015).

Oryzagenome (http://viewer.shigen.info/oryzagenome/) is a recently developed SNP information viewer for all genome types included in the genus *Oryza* (Ohyanagi et al. 2016). In the current version (Release 1.0), there are genome sequences of 21 wild *Oryza* species and several cultivars. The SNP information obtained from 446 accessions of *O. rufipogon* is also available. As the genome and related omics data accumulate, this type of database will become increasingly important for integrated omics studies.

28.5.1 Future Perspective

Since genomics and other omics became popular in the 1990s, people also started to recognize the value of databases in which large-scale omics data can be stored. In fact, as the importance of the databases increased, so did the publication of such databases. This trend led to the foundation of a journal specialized for biological databases (Landsman et al. 2009). Additionally, some scientific editors were motivated to dedicate an entire issue to databases (Galperin et al. 2017). Later, some other journals followed this trend and created a similar issue (Matsuoka 2011) or section (Kumar 2013) for databases, and currently many biological journals have resources that include databases within its scope. In this way, database-related studies form one of the hottest areas in biology at the moment. One may suspect that this type of scientific framework, which is supported by the traditional peer-review system, would soon become outdated for the presentation of large-scale biological data. Database papers generally describe an outline of the databases and their contents, which are frequently updated without any additional publications. Currently, not only analyzed omics data but also untreated high-throughput data are immediately available; these data can be coupled or uncoupled with traditional-style publication, and there is also an online system to support publication of such a gigantic data set (Goodman et al. 2012). Many of the databases may function as repositories where the data are stored and released even before publication.

It is the increase in DNA and RNA sequences that has accelerated bioinformatics. As a result, the databases that harbor the computationally processed sequence data created by bioinformatics were needed. However, we must note that omics data are not restricted to sequence information. Other omics, such as proteomics, metabolomics, and interactomics, are also the subjects of large-scale molecular biology. Nonetheless, the omics databases of rice are currently biased toward genome and transcriptome data, while data of other omics, such as proteome and metabolome, are scarce. The reason is probably because, so far, omics applications in crop sciences have been related mainly to the areas of genomics or transcriptomics that can be used for marker-assisted selection, genome-wide association studies, and genomic selection. Sequence information that is linked to other resources should be the first priority for rice and other crops for the time being, but

we expect that other omics data will increasingly be necessary for next-generation breeding. Thus, databases that provide users with comprehensive resources, such as Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/) (Kurata and Yamazaki 2006) and Gramene (Tello-Ruiz et al. 2016), will be of great importance as a hub for rice omics data that will make in-depth analyses possible.

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