Chapter 22
Genetic Dissection and Breeding for Grain Appearance Quality in Rice

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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

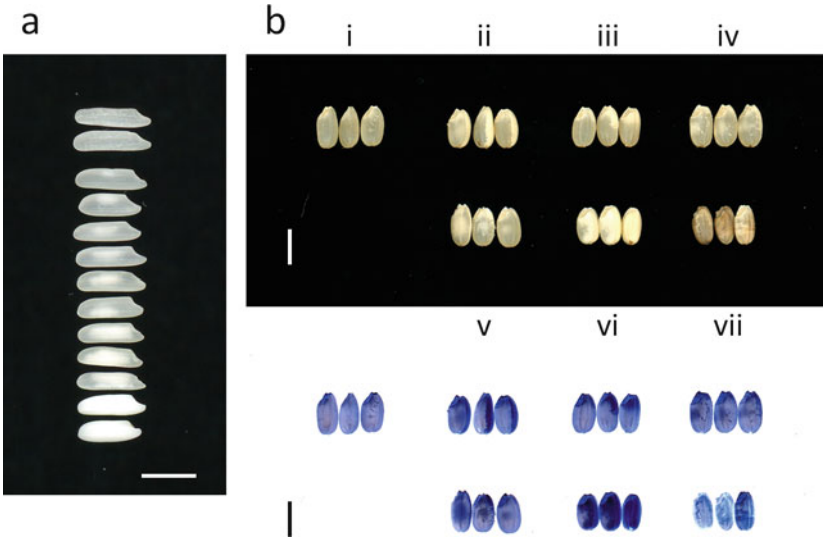

Fig. 22.1 Grain appearance in rice cultivars. (**a**) Chalky grains of *indica* cultivars. (**b**) Chalky grains of *japonica* cultivars: (*i*) normal (no chalkiness), (*ii*) white-back, (*iii*) white-base, (*iv*) white-belly, (*v*) white-core, (*vi*) milky-white, and (*vii*) abortive in normal color (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.

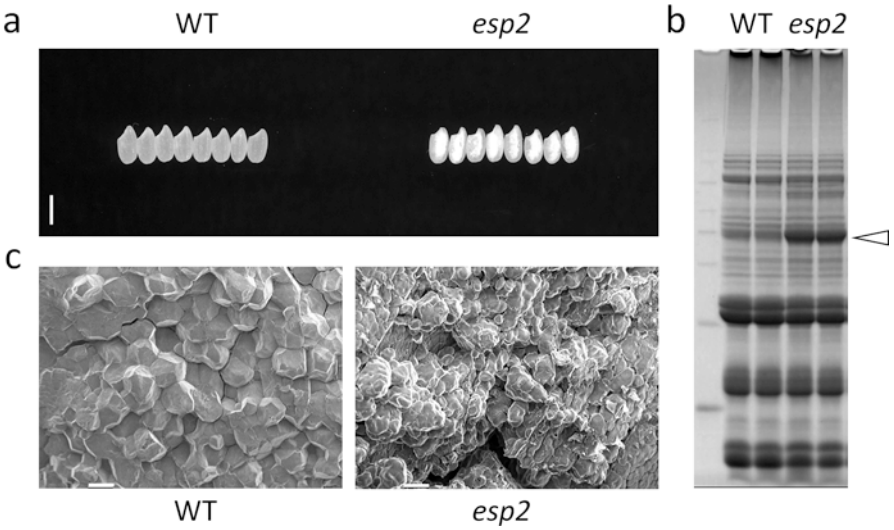

Fig. 22.2 Phenotypes of rice grains in wild type (WT) and *esp2* mutant. (**a**) Appearance of polished rice grains. Scale bar, 5 mm. (**b**) SDS-PAGE image of storage proteins in mature seeds. Arrow indicates accumulation of glutelin precursor in the *esp2* mutant. (**c**) Scanning electron microscopy 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

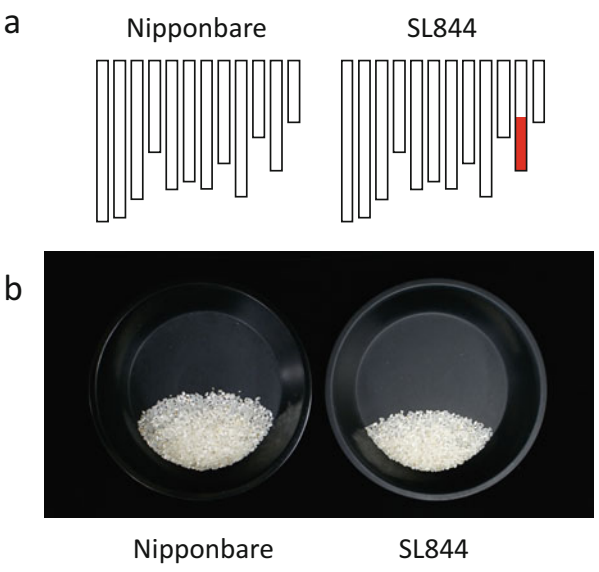

Fig. 22.3 Milling quality of Nipponbare and SL844, with a Koshihikari chromosome fragment (red) in the Nipponbare genetic background (white). (**a**) Graphical genotypes representing the 12 rice chromosomes. (**b**) Abortive and cracked grains after milling 1.5 kg of brown rice

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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