Functional analysis of genes involved in the regulation of development of reproductive organs in rice (Oryza sativa)

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Chapter 1

Molecular and environmental determination of grain quality in rice (*Oryza sativa*)

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Introduction

Food supply is still a serious problem when we started the journey into a new millennium. According to the FAO (Food and Agriculture Organization of the United Nations) report "The State of Food Insecurity in the World 2009" (http://www.fao.org/docrep/012/i0876e/i0876e00.htm), the undernourished population worldwide has surpassed one billion people for the first time in the history of mankind. Rice amongst other cereal grasses like millet, maize, barley, sorghum, wheat and rye is one of the oldest cultivated food crops and consumed in many countries. It is the key to food security of at least half the world population. The final purpose of rice breeding is to produce cultivars with more yield as well as higher nutrient quality and displaying yield stability under biotic (diseases) or abiotic stress (e.g. drought, salt, flooding and lodging). Since the 1960s, the productivity of rice has been largely improved due to the so-called Green Revolution by the development of inbred and hybrid cultivars, irrigation infrastructure, modern management techniques, synthetic fertilizers and pesticides. However the nutritional value of grains with respect to protein content and micro-elements is still behind our expectations. Therefore, novel insights underlying the mechanisms which regulate different aspects of grain quality in rice are not only a main subject for scientific research but also of utmost importance for breeding.

Rice grain qualities and preference vary across regions, but most interests are widely shared (Efferson, 1985; Unnevehr et al., 1985; Cheaupun et al., 2004). Appearance, nutritional value, and cooking quality are the principal characters concerned by producers and consumers (Yu et al., 2008); and milling is another important factor to consider for farmers and processing industry. Relevance of the traits determining grain quality is slightly different among different countries. However, several parameters are of common interests for the four major issues listed above. Appearance is mainly associated with grain size, shape, chalkiness, and translucency. Amylose content, protein content, gelatinization temperature and gel consistency determine cooking quality. Protein content is also a decisive parameter for the nutritional value, in that rice grain contains 5-12% of storage protein in the endosperm (Villareal and Juliano, 1978). Three standards are widely used to define milling quality, namely brown rice rate, milled rice rate and head rice rate, which represent the ratios of grains produced out of different intensity of milling from just dehusked to removing all of the bran and germ.

The quality of rice grain depends on the composition of storage starch and protein content

Rice accumulates two types of starch, amylose and amylopectin. The major storage proteins in rice can be grouped into four types based on their physical properties of solubility, i.e., water-soluble albumin, salt-soluble globulin, alkaline-soluble glutelin and alcohol-soluble prolamin. Most characteristics of grain quality in rice are decided directly or indirectly by type and content of starch and protein in the endosperms. This suggests that many traits are mutually correlated and influenced by each other. For instance, milling reduces protein and fat content while increases whiteness and thus reduces the nutrient value. However, most of the consumers prefer pseudo-cosmetic preferences and demand white or polished rice. Increase of milling decreases the gelatinization temperature in that removing of the outer part of the brown rice facilitates the absorption of water (Champagne et al., 1990; Muramatsu et al., 2006). It also improves flavour, hardness, chewiness and adhesiveness of cooked rice (Park et al., 2001; Saleh and Meullenet, 2007). On the contrary, higher seed storage

protein content prevents breakage of the endosperm during milling (Leesawatwong et al., 2004). Chalkiness is related to the shape, size and packing of amyloplasts which are organelles responsible for the synthesis and storage of starch granules within endosperms. Translucent rice kernels from different cultivars have significantly higher amylose content than chalky grains (Rani and Bhattacharay, 1989; Lisle et al., 2000; Singh et al., 2003). Furthermore, the amylose content is the largest determinant of cooking and eating quality of the milled rice (Juliano and Villareal, 1993). Comparison of chemical compositions and physicochemical properties in different varieties revealed that amylose content is significantly correlated with texture and gel properties of cooked rice (He and Suzuki, 1987; Sowbhagya et al., 1987; Rani and Bhattacharay, 1989; Ong and Blanshard, 1995; Singh et al., 2003; Cameron and Wang, 2005; Allahgholipour et al., 2006; Kibanda and Luzi-kihupi, 2007). Besides amylose, the structure of amylopectin is another crucial factor for cooking in that the ratio of short and long chains of amylopectin affects gelatinization temperature and texture after cooking (Ong and Blanshard, 1995; Ramesh et al., 1999; Mizukami and Takeda, 2000; Cameron and Wang, 2005; Nakamura et al., 2006). In addition, cooking quality is also affected by seed storage protein. It was suggested that protein-starch interactions could impede starch gelatinization, and disruption of the structure of proteins can increase viscosity of cooked rice (Hamaker et al., 1991; Hamaker and Griffin, 1993; Cameron and Wang, 2005; Derycke et al., 2005; Yu et al., 2008). In short, most of the grain quality traits in rice are decided by the content and composition of storage starches and seed storage proteins in the endosperm. Thus the development of endosperm is crucial in determination of the grain quality.

Storage starch and protein accumulation in endosperm

Rice caryopsis is developed from the fertilized pistil. Figure 1 shows the internal structure of a rice grain. Enveloped by the brownish pericarp, the dehulled grain is called brown rice. Next to the pericarp are two layers of cells named tegmen or seed coat. The embryo lies on the ventral side of the spikelet next to the lemma. The remaining part of the caryopsis is the endosperm which provides nourishment to the germinating embryo. The embryo contains a plumule (embryonic leaves) and radicle (embryonic root), which are joined by a very short stem (mesocotyl). The portion tied to endosperm forms the scutellum. The endosperm is wrapped by the aleurone layer beneath the testa (seed coat), having the starch storage parenchyma inside (Chang et al., 1965; Matsuo and Hoshikawa, 1993). Grain development is a continuous process and the grain undergoes distinct changes before it fully matures. In the tropics, the ripening stage takes 25-35 days regardless of the cultivar and in temperate countries needs 45-65 days. Ripening involves progressive stages, termed milky, dough, and mature grain stage (DeDatta, 1981). After fertilization, endosperm cells proliferated and differentiated, lasting about 12 days. The dry matter such as starch and protein is accumulated strongly from three day after flowering (DAF) to DAF12 (Rosario et al., 1968; Xiong et al., 2005). About ten DAF, the internal part of the endosperm starts to become translucent and harden starting from the center and then proceeds to surrounding portions (Matsushima and Manaka, 1956). The endosperm cells of the translucent part are filled densely with starch and other reserve substances; however the opaque part is not completely filled and has many minute gaps (Matsuo and Hoshikawa, 1993).

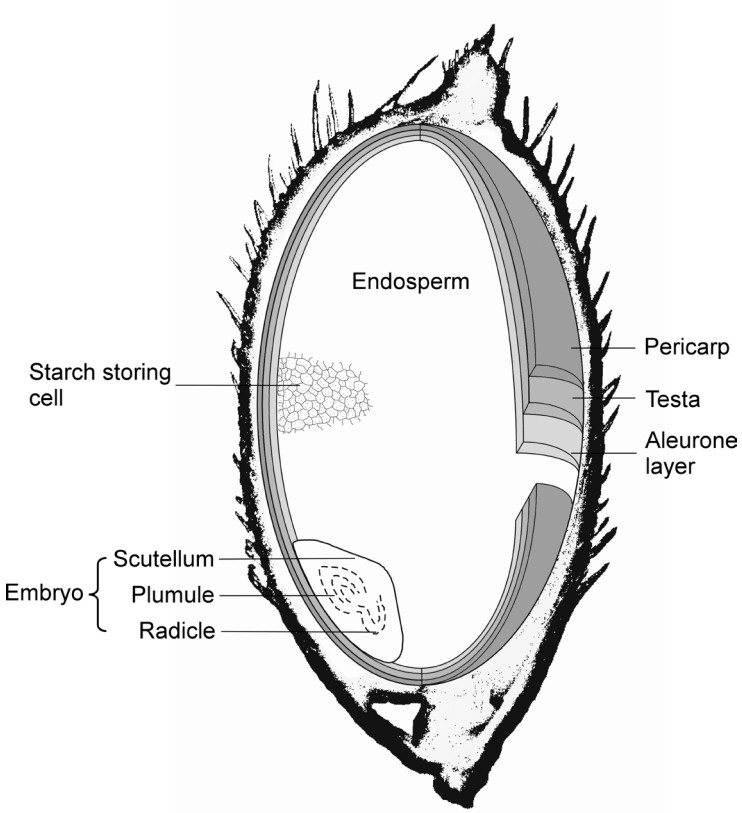

Figure 1. Structure of a rice grain

Rice grains deposit starch in particles. Four days after anthesis many tiny proplastids appear in the cytoplasm in the innermost cells of the endosperm tissue and it is in these proplastids that starch granules emerge. Some of the starch grains in the original proplastids move to the new proplastids formed by division, but new starch granules are also formed in the new proplastids. Two to three days after their appearance, proplastids stop multiplication and each of them begins to grow larger. The starch granules within them also multiply in number. The developed plastids are then known as amyloplasts. The surface of amyloplasts is covered with a thin double membrane and the outer membrane is linked to the endoplasmic reticula (ER) and Golgi apparatuses in cytoplasm. With the development of the seeds, starch grains develop and store from inner to outer cells of the endosperm. At 15 DAF, the innermost cells are filled with starch grains and complete their growth in size. The completion of starch grain development gradually spreads to the peripheral areas and ends by about 30-35 DAF (Matsuo and Hoshikawa, 1993).

Unlike the other crops, up to 80% of the total storage protein in rice is glutelin (Yamagata et al., 1982) and prolamin takes 20 to 30%.Thus the composition and structure of glutelin and prolamin have the decisive role in determining the nutritional traits in rice. Similar as starch, reserve protein occurs as grains in the endosperm. Protein bodies (PB) in rice can be divided into two groups, PB-I and PB-II, according to their morphologically characteristics. Both PB-I and PB-II are distributed throughout the rice endosperm, especially rich in aleurone and subaleurone layers. Protein grains can be observed first on the sixth to seventh days after pollination as the forms of proteoplasts which

contain small spherical protein bodies. Proteoplasts gradually increase until the late ripening stage and are distributed more in the peripheral parts of the endosperm (Matsuo and Hoshikawa, 1993). Although four storage proteins are all initially synthesized on the ER membrane and translocated into the ER lumen, they are stored in different protein bodies. Glutelins are transported to the protein storage vacuole by way of the Golgi and form PB-II. Prolamines, on the other hand, are assembled and deposited within the lumen of the rough ER to form PB-I (Ogawa et al., 1987; Li et al., 1993b; Muntz, 1998). PB-I is spherical, with a diameter of about 1 to 2 μm, and exhibits concentric rings of varying electron density. In contrast, PB-II is larger (3-4 μm), irregularly shaped, and of highly uniform staining density (Krishnan and White, 1995; Takemoto et al., 2002).

Molecular basis of grain quality in rice

Comparison of quality traits revealed significant variation among cultivars and varieties grown in the same environment (Adu-Kwarteng et al., 2003; Cameron and Wang, 2005; Kang et al., 2006; Vidal et al., 2007). Hence, the decisive factors controlling the grain quality lie in the rice genome itself, i.e. in the loci encoding starch synthetases and storage proteins. Regulation of these genes on both transcription and post-transcription levels may also play important roles. Furthermore, studies on genetic diversity in starch synthetases revealed essential markers which affect different traits. Besides that, mapping-based methods provided new ways of searching for important genes involved in regulation of grain quality. Recent studies show that many traits are not only determined by single genes but also influenced by quantitative trait loci (QTLs). Although many of the QTLs are defined in comparatively wide genomic regions, some major effects have been identified including the effect of the *Waxy* (*Wx)* gene. In following sections, the knowledge so far of molecular basis for grain quality of all the aspects above was summarized.

Starch synthetases determine the content and characteristics of amylose and amylopectin in rice endosperm

Starch in the endosperm of rice is the dominant form of carbon reserves in grains. Biosynthesis of starch in rice grains start from degrading of sucrose, the main transported form of assimilates in rice. The process of sucrose to starch involves a series of enzymes. All enzymes in rice have different isoforms and are encoded by multiple genes, leading to a highly complex biosynthesis and accumulation process. Mutant studies in the past thirty years have revealed the function of many of these enzymes and their involvement in the quality of rice grain.

Sucrose synthase (SUS) is responsible for the first step in starch biosynthesis by catalyzing a reaction of cleaving sucrose into UDP-Glucose and D-Fructose. The native form of the enzyme is a tetramer composed of subunits with a molecular mass of about 90 kDa. In rice there are four different isozymes in different tissues (Chan et al., 1990). Three genes (*RSus1, 2 and 3*) have been cloned and their expression patterns were characterized (Wang et al., 1992a, 1999; Wang, M.B. et al., 1992; Yu et al., 1992; Huang et al., 1996; Odegard et al., 1996). It appears that *RSus2* is ubiquitously expressed in both vegetative tissues and seeds. *RSus3* is highly specific to the grain, and the levels of *RSus1* and *RSus3* in developing seeds are closely complementing one another, both spatially and temporary (Huang et al., 1996; Wang et al., 1999). Till now no mutants of these genes have been reported in rice. It may be because loss function of RSus2 is lethal for the plants and knock out of

the other two can be compensated by the function of RSus2.With the development of more mutant populations, mutants for theses three genes might be available in the future.

After degrading of sucrose, four types of enzymes are generally accepted playing the key roles in synthesis of starch in cereals: ADP Glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE) and starch debranching enzyme (DBE) (reviewed in (James et al., 2003)). AGPase produces the activated glucosyl donor ADP-glucose that is the primer of the starch chain and is regarded as the rate-limiting enzyme in starch biosynthesis. AGPase in rice has large and small subunits which encoded by four and two genes respectively (Jeon et al., 2010). Mutants of *OsAGPL2* and *OsAGPS2b*, genes encoding the large and small subunits respectively, show a shrunken endosperm due to a severe reduction in starch synthesis (Lee et al., 2007).

SS, composed of both soluble and granule-bound isoforms, catalyzes the chain-elongation reaction of α-1,4-glucosidic linkage by transferring a glucose moiety from ADP-glucose to the non-reducing end of the linkage. Granule-bound SS (GBSS) and soluble SS (SSS) are responsible for the synthesis of amylose and amylopectin respectively. GBSS in rice has two isoforms expressed in storage (GBSSI) and none-storage (GBSSII) tissues. GBSSI is also called Wx and its role in biosynthesis of amylose is very well defined. The storage starch in non-waxy varieties is composed of 15% to 30% amylose and 70% to 85% amylopectin (Umeda et al., 1991); however the *wx* mutant endosperm contains almost exclusively amylopectin. *Wx* genes have been cloned in *O. sativa* (Okagaki and Wessler, 1988; Wang et al., 1990; Hirano and Sano, 1991; Okagaki, 1992) and *O. glaberima* (Umeda et al., 1991). Two wild-type alleles, *Wxa* and *Wxb*, were found at the *waxy* locus in cultivated rice. *Wxa* is characteristic of indica rice, and *Wxb* is found mainly in japonica rice (Sano, 1984; Sano et al., 1986). A naturally occurring single base mutation at the 5’ splicing site of the first intron differing *Wxa* and *Wxb* alleles is the direct reason behind the expression polymorphism of *Wx* mature mRNA between indica and japonica rice (Hirano et al., 1998; Isshiki et al., 1998). Thus the low expression of the gene in japonica rice results in more sticky rice and the high expression in indica rice results in less sticky rice when cooked (Hohn and Puchta, 2003).

Synthesis of amylopectin is more complicated than amylose and involved all four types of enzymes including AGPase, SSS, SBE and DBE (reviewed in (Nakamura, 2002)). SSS in rice has eight isoforms, namely SSI, SSIIa (SSII-3), SSIIb (SSII-2), SSIIc (SSII-1), SSIIIa (SSIII-2), SSIIIb (SSIII-1), SSIVa (SSIV-1), SSIVb (SSIV-2) (Hirose and Terao, 2004). SBE generate α-1, 6 linkages by cleaving internal α-1, 4 bonds and transferring the released reducing ends to C6 hydroxyls. Thus forms branches on polymers. DBE is capable of removing some of these branches. SBE contains three isoforms (BEI, BEIIa and BEIIb) in rice. Physiochemical analysis of the mutants revealed the various roles of SSI (Fujita et al., 2006), SSIIa (Umemoto et al., 2002; Umemoto et al., 2004; Nakamura et al., 2005), SSIIIa (Fujita et al., 2007), BEI (Satoh et al., 2003), BEIIa (Nakamura, 2002), BEIIb (Nishi et al., 2001; Tanaka et al., 2004) in the synthesis of different chains of amylopectin in recent years. DBE has two forms, isoamylase and pullulanase. The genes encoding isolamylase (Fujita et al., 1999) and pullulanase (Nakamura et al., 1996) have been cloned. Although the role of DBE used to be restricted to the debranching of amylopectin during complete hydrolysis of starch, studies on the mutants *sugary 1* and isolamylase antisense transgenics in last two decades revealed the involvement of isoamylase and pullulanase in the synthesis of amylopectin in rice (Nakamura et al., 1996, 1997; Kubo et al., 1999; Fujita et al., 2003, 2009). Besides these enzymes, Wx also bears the potential capability in formation of extra-long unit chains (ELCs) of amylopectin

in the SSIIIa-deficient mutant, which has a high level of ELC amylopectin and pleiotropically increased Wx in the endosperm (Fujita et al., 2007). This function was later verified using transgenic plants by transforming the *Wx* gene into the null-mutant *wx* rice (Hanashiro et al., 2008).

Alteration of amylopectin content and structure has significant impact on the morphology of starch granules and eventually affects cooking and consumption traits. It has been reported that silencing of *SSIIIa* (Fujita et al., 2007; Ryoo et al., 2007), *BEI* (Satoh et al., 2003), *BEIIb* (Nishi et al., 2001), isoamylase gene *OsISA1* (Fujita et al., 2003), changes the gelatinization properties of the starch endosperm. In another study the indica *SSIIa* gene, which carries two variations in amino acids and thus affects the activity of the enzyme, was introduced into japonica rice which enabled japonica rice cultivars to synthesize indica-type amylopectin. The starch in the transformed japonica rice exhibited gelatinization-resistant properties that are characteristic of starch from Indica rice (Nakamura et al., 2005).

Besides starch synthetases, plastidial starch phosphorylase Pho1 also has important roles in amylopectin synthesis. Loss of *Pho1* caused smaller starch granules to accumulate and modified the amylopectin structure. The size of mature seeds and the starch content in *pho1* mutants showed considerable variation, ranging from shrunken to pseudonormal (Satoh et al., 2008).

Protein compositions in rice grains are mainly decided by genes encoding glutelins and prolamins

Glutelin in rice is synthesized as a 57 kDa precursor and then cleaved into a 37 to 39 kDa acidic subunit and a 22 to 23 kDa basic subunit (Yamagata et al., 1982). Encoded by about 15 genes per haploid genome, glutelin genes can be classified into four subfamilies, GluA, B, C and D. GluA contains four members and GluB has the highest with eight members. Thus far, only two members of GluC and one member of the GluD subfamily have been identified (Takaiwa, 1987; Okita et al., 1989; Takaiwa et al., 1991; Mitsukawa et al., 1998; Kusaba et al., 2003; Katsube-Tanaka et al., 2004; Kawakatsu et al., 2008). As the second abundant protein in rice endosperm, the prolamins have molecular masses of ranging from 12 to 17 kDa. The rice prolamins are encoded by a complex multigene family with 80 to 100 copies per haploid genome (Kim and Okita, 1988a, b; Masumura et al., 1989; Barbier and Ishihama, 1990; Feng et al., 1990; Masumura et al., 1990; Shyur and Chen, 1990; Horikoshi et al., 1991; Shyur et al., 1992; Yamagata et al., 1992; Wen et al., 1993; Mitsukawa et al., 1999).

Because of the redundancy of glutelin and prolamin genes in rice, it is difficult to identify naturally occurring varieties with obviously altered storage protein levels. The only report so far is a selection of 19 varieties among 1,400 accessions, from Russia, Northern China and North Korea, showing an increased content of the 57 kDa glutelin precursor with markedly decreased content of acidic and basic subunits (Satoh et al., 1995). However, uncovering the molecular basis behind this qualitative trait is challenging and time consuming. Gamma-ray irradiation and chemical mutagen treatment provide alternative approaches and have been used to create mutants with variable glutelin and prolamin compositions (Kumamaru et al., 1988; Iida et al., 1993, 1997). A mutant line *NM67* obtained from a collection of 1,433 mutated lines of cultivar ‘Nihonmasari’ treated with 0.2% ethyleneimine, was found to have a lowered grain content of glutelin and a higher content of prolamine. Genetic analysis revealed that low glutelin content was always accompanied by high prolamine and this phenemenon seemed to be manifested by a single dominant gene. An improved

low glutelin and high prolamine line (LGC-1) has been developed from a backcross between *NM67* and its original cultivar (Iida et al., 1993). Another nine mutant lines lacking glutelin subunits were selected from M2 seeds of about 10,000 M1 plants mutagenized with gamma rays or ethylmethanesulfonate and from 1,400 mutant lines selected originally for morphological characters. Three types of mutants were identified. One line lacks the largest subunit among four minor bands of glutelin acidic subunits. Five lines lack the second largest subunit band, and three lines do not have the third largest subunit band. Genetic analysis of the mutated genes showed that these mutant characters were controlled by single recessive genes named *glu-1*, *glu-2*, and *glu-3*, respectively (Iida et al., 1997). LGC-1 and *glu-1* exhibited the same phenotype, indicating they may have the similar genetic basis. Further research confirmed this hypothesis. In LGC-1 homozygotes, there is a 3.5-kb deletion between two highly similar and tandem repeated glutelin genes *GluB-5* and *GluB-4* that forms a tail-to-tail inverted repeat, which may produce a double-stranded RNA molecule and induced RNA silencing (Kusaba et al., 2003). On the other hand, *glu-1* harbours a 129.7-kb deletion which eliminates the entire *GluB-5* and *GluB-4* gene except half of the first exon of *GluB-5* (Morita et al., 2007). Comparison of LGC-1 with cultivars with normal glutelin content by light microscopy and confocal laser scanning microscopy revealed that low-glutelin rice differs from the other cultivars not only in the major storage protein composition but also in the distribution of proteins in endosperm tissues (Furukawa et al., 2003).

Expression of starch synthetase and seed-storage protein genes is overlapping

Most of the starch synthetase genes expressed during grain filling of rice follow a highly similar expression style. The mRNA transcripts increase with the development of seeds till peaked at either six or ten DAF, and then decline gradually towards maturation. The only exception was the expression of the *Wx* gene which was initiated at three DAF. After reaching a peak activity at six DAF, the expression of *Wx* declined towards ten DAF but rose again and peaked at 15 DAF before declining to trace level at 20 DAF (Duan and Sun, 2005). The expression pattern of *Wx* in rice grain is shown to be cultivar dependent (Wang et al., 1995).

On the other hand, rice glutelin and prolamin genes are expressed exclusively during seed development. Similar to starch synthetases, the majority of the seed-storage protein genes in rice are developmentally regulated. A recent profiling study of six members from the GluA and GluB subfamilies showed that none of them had any detectable expression level at three DAF but with a peak activity at 10 DAF before declining towards maturation (20 DAF) (Duan and Sun, 2005; this thesis). This common pattern was also discovered in other studies on GluA, GluB and GluC subfamily members (Okita et al., 1989; Takaiwa and Oono, 1990, 1991; Mitsukawa et al., 1998). *GluD-1* however is different from the other glutelin genes. It is detectable in seed tissue at five DAF with increasing intensity through 30 DAF (Kawakatsu et al., 2008). As described in previous section, rice prolamins are encoded by 80 to 100 copies of genes. Among them, a 10 kDa prolamine gene and three members of the 13 kDa prolamine family followed a largely identical expression pattern compared to glutelin genes. However the other two genes from 13 kDa subfamily showed a distinct expression pattern, in that they were expressed increasingly, but at low level, towards seed maturation (20 DAF) (Duan and Sun, 2005). The transcripts of two 16 kDa prolamin genes also showed a gradually rising way and start to accumulate from eight DAF to the maturation of rice seeds till 26 to 28 DAF (Shyur et al., 1992; Mitsukawa et al., 1999).

Common *cis*-regulatory elements exist in regulatory regions of starch synthetase and seed-storage protein genes

All seed-storage protein and starch synthetase genes are strongly active during the development of the grains, indicating they may share similar regulation mechanism on the transcription level. Indeed several universal *cis*-regulatory elements have been identified in the 5' upstream region of these genes, not only in rice but also in other crops. Comparisons of the promoter sequences of cereal prolamin genes have identified a conserved region at ~300 bp upstream of the transcriptional start. This so-called bifactorial endosperm box is composed of two closely located motifs, a prolamine box class endosperm motif and a GCN4-like motif (Kreis et al., 1985). GCN4 and prolamin boxes have been discovered in promoters of seed storage protein genes in wheat, maize, sorghum, barley, wheat, ray and oat (Colot et al., 1987; Albani et al., 1997; Vicente-Carbajosa et al., 1997; Marzábal et al., 1998; Norre et al., 2002). Another two common motifs are AACA and ACGT boxes. GCN4, AACA, and the prolamin box were found in GluA, GluB and GluD family members in rice. ACGT motif also exists in these genes except for *GluA-1* (Takaiwa et al., 1996; Yoshihara and Takaiwa, 1996; Yoshihara et al., 1996; Washida et al., 1999; Wu et al., 2000; Kawakatsu et al., 2008; Qu le et al., 2008). However, the GluC promoter did not contain these *cis*-elements which means it has its own unique way of regulation (Qu le et al., 2008). Prolamin box and GCN4 motif are also identified in rice prolamin genes (Zhou and Fan, 1993; Wu et al., 1998; Su et al., 2001). Compared to seed storage protein genes, studies on the promoter regions of starch synthetases are poor. However, GCN4 and prolamin boxes are also found in regulatory regions of the *Wx* gene in rice (Cheng et al., 2002) and in the gene for the starch branching enzyme *sbeIIa* in wheat (Miao et al., 2004). Considering the equivalent expression pattern of starch synthetase genes (Duan and Sun, 2005), these elements might be also localized within the 5' regulatory region of other starch synthetase genes.

Transcription regulators of starch synthetases and seed-storage proteins

Despite the increasing number of studies and report on the activities of different classes of *cis*-elements controlling seed-specific gene expression, identification of the corresponding trans-acting factors in rice and other cereals are still limited. One of the earliest known transcription factors specifically involved in grain development is Opaque2 from maize (Schmidt et al., 1992). In rice, a basic leucine zipper family (bZip) protein RITA-1 was identified first to be able to bind to ACGT element and activate reporter gene expression in transient assays (Izawa et al., 1994). Another bZip protein REB was found later to interact specifically with the GCCACGT(c/a)AG sequence in the α-globulin promoter (Nakase et al., 1997). In more recent studies, five different bZip proteins named RISBZ1 to RISBZ5, two of them RISBZ2 and RISBZ3 were completely identical with RITA-1 and REB, were identified in a cDNA library derived from rice seeds. They were able to bind to the GCN4 motif from the rice *GluB-1* promoter but only RISBZ1 is capable of *trans*-activating the expression of a reporter gene preceded by a minimal promoter fused to a pentamer of the GCN4 motif (Onodera et al., 2001; Kawakatsu et al., 2008). The AACA sequence in glutelin gene promoters is the target site for the Myb domain factor OsMYB5 (Suzuki et al., 1998). The Dof (DNA binding with one finger) prolamin box binding factor (RPBF) is able to recognize AAAG/CTTT motifs in the *GluB-1* promoter. RISBZ1 and RPBF both can *trans*-activate GUS activity driven by promoters of different storage protein genes in transient assays, such as *GluA-1*,

GluA-2, GluA-3, GluB-1, GluD-1, *10 kDa Prolamin, 13 kDa Prolamin, 16 kDa Prolamin,* and *α-Globulin*. Synergistic interactions between RISBZ1 and RPBF were also discovered in transient assays. In our laboratory two Dof proteins, OsDof24 and OsDof25 were also found to be able to specifically interact with AAAG/CTTT motifs (Zhang Yu et al, thesis in preparation). However, much of the evidence so far for the biological functions of these transcription factors is from gain-of-function experiments in transient assays. Our group tried to identify T-DNA or transposon tagged mutants for several transcription factors. Unfortunately, no obvious phenotype and alteration of grain starch and storage protein content have been discovered. This may be due to the redundancy of the transcription factors and the multi-copy number of the starch synthetase and storage protein genes and which make any future research on this topic difficult and challenging. Till now the evidences in rice which showed alteration in target gene expression and protein accumulation were only discovered in knock-down transgenics of both RISBZ1 and RPBF by RNA silencing (Yamamoto et al., 2006; Kawakatsu et al., 2008, 2009).

For starch synthetase genes there are even less transcription factor genes regulating their expression known than for the seed-storage protein genes. The rice bZip protein REB can specifically interact with the GCN4 motif in promoter of the *Wx* gene as well as bind to the target sequence in the promoter of α-globulin (Cheng et al., 2002). OsBP-5, a Myc protein binds specifically to a 31-bp sequence of the 5' upstream region of the *Wx* gene and can *trans*-activate gene expression synergistically with the EREBP family protein OsEBP-89 by forming a heterodimer (Zhu et al., 2003). The recessive *floury-2* (*flo-2*) locus of rice causes a strong reduction in *Wx* and AGPase expression but also in expression of the genes encoding two isoforms of starch branching enzyme in developing seeds (Kawasaki et al., 1996). This indicates that co-regulation by a common *trans*-acting regulator does occur for different starch synthetases genes.

Post-transcriptional regulation

Regulation of starch synthetase and seed-storage protein genes does not only occur on the transcriptional level but also post-transcriptionally. Analysis of a collection of 31 rice varieties for levels of *Wx* transcript, Wx protein and amylose revealed that the levels are correlated with the ability to excise intron 1 from the leader sequence of the *Wx* mRNA transcript. Nucleotide sequences of the *Wx* intron 1 in different cultivars differ by 16 bases. These altered nucleotides contribute to the improper splicing and incomplete splicing of intron 1 in certain cultivars. As a consequence, the total amount of translatable *Wx* mRNA, and therefore the Wx protein and consequently amylose content, are reduced (Wang et al., 1995; Cai et al., 1998). Later studies further confirmed the importance of specific sequences in *Wx* intron 1 associated to the correct splicing of the pre-mRNA (Ayres et al., 1997; Hirano et al., 1998; Larkin and Park, 1999; Isshiki et al., 2000; Mikami et al., 2000; Larkin and Park, 2003; Bao et al., 2006a; Prathepha, 2007).

The expression of the glutelin and prolamin multigene families is not coordinated but instead are differentially regulated on not only the transcriptional but also post-transcriptional levels. Although approximately 4-fold more abundant than the prolamines on a weight basis due to their higher molecular mass in the mature seeds, glutelins are only slightly more abundant than prolamines on a molar basis. Both storage proteins were first detected in 10 DAF seeds and their amounts were steadily increasing throughout seed development, but the protein and mRNA levels of glutelins and prolamines are not constant at different stages of grain development. The molar ratio of

glutelin to prolamine proteins was 1.7 in ten day old seeds (Li and Okita, 1993). However equal amounts of glutelin and prolamine mRNAs were found in five and ten day old seeds (Kim et al., 1993). The glutelin and prolamine protein ratio steadily decreased to 1.2 in 25 day old seeds due to the increased synthesis and accumulation of prolamines specifically during the latter stages of seed development (Li and Okita, 1993), which was consistent with the mRNA levels change. A 40% excess of prolamine transcripts, relative to glutelin transcripts, was discovered in seeds of 15 days and older. Quantification of total mRNA levels revealed equal amounts of glutelin and prolamine mRNAs in five and ten day old seeds. But a 40% excess of prolamine transcripts, relative to glutelin transcripts, was discovered in seeds of 15 days and older (Kim et al., 1993). These evidences suggest that post-transcriptional regulation is most likely involved in the synthesis of glutelins and prolamines at early stage.

Moreover, ER associated translational control is an important process affecting storage protein synthesis. In the cells of developing rice endosperm, the mRNA of prolamins and glutelins has been shown to be associated with different areas of the rough ER. Glutelin mRNAs are enriched approximately 2.5-fold more than the prolamine mRNAs on the rough cisternal ER membranes, whereas the prolamine mRNAs are 7- to 10-fold more abundant than the glutelin mRNAs on the rough ER membranes that surround the prolamine PBs (Li et al., 1993a). This might represent the first step in the sorting of the two different classes of seed-storage proteins. Evidence of ER involved regulation on post-transcriptional level is further supported by the analysis of *esp2* mutants which contain higher levels of the 57 kDa polypeptide and correspondingly lower levels of acidic and basic glutelin subunits than normally. Electron microscopic observation revealed that *esp2* contained normal PB-II, but lacked the normal PB-I. Instead, numerous small ER-derived new PBs that contained the 57 kDa glutelin precursor and prolamin polypeptides were observed. These proteins form glutelin-prolamin aggregates via interchain disulfide bonds within the ER lumen. The endosperm of *esp2* mutants contains the lumenal chaperones, binding protein and calnexin, but lacks protein disulfide isomerase (PDI) at the protein and RNA levels. These results suggest that PDI plays an essential role in the segregation of proglutelin and prolamin polypeptides within the ER lumen (Takemoto et al., 2002). The 57 kDa glutelins precursors are sorted into protein storage vacuoles where they are processed into acidic and basic subunits. Recent work demonstrated that processing of proglutelin by vacuolar processing enzyme in rice is essential for proper protein storage vacuole structure and compartmentalization of storage proteins (Wang et al., 2009; Kumamaru et al., 2010).

The cytoskeleton is also known to be involved in synthesis of seed-storage proteins. Biochemical studies have shown that prolamine mRNAs may be anchored to the surface of prolamine PBs via the cytoskeleton (Muench et al., 1998). Confocal microscopy of endosperm cells further revealed that, unlike the glutelin PBs, the developing prolamine PBs are not randomly distributed within the cell, but instead are often enriched in the cortical region of the cell only a few micrometers beneath the plasma membrane. In addition, the peripherally localized prolamine PBs are in very close juxtaposition with the cortical microtubule and actin filament networks (Muench et al., 2000).

Genetic diversity in starch synthetase genes is related to the physicochemical properties of rice grain

Besides the *cis*-acting elements and *trans*-acting factors, genetic diversity within the genomic regions of the starch synthetase genes also showed regulatory effects of quality traits in rice. The most well studied molecular marker is the single nucleotide polymorphisms (SNP) in the first intron of *Wx* gene which is involved in the proper splicing of *Wx* mRNA. The natural G to T change in this locus results in two alleles *Wxa* and *Wxb* which determine significantly different amylose content (Wang et al., 1995; Ayres et al., 1997; Cai et al., 1998; Hirano et al., 1998; Mikami et al., 2000; Larkin and Park, 2003; Han et al., 2004; Bao et al., 2006a; Prathepha, 2007). Another intensively studied polymorphism is a microsatellite (SSR) locus of $(CT)_n$ repeats identified in the 5'-untranslated region of the *Wx* gene (Bligh et al., 1995). Various alleles of this locus were identified in non-waxy cultivars which associate with the variation in amylose levels (Ayres et al., 1997; Bergman et al., 2001; Tan and Zhang, 2001; McClung et al., 2005; Bao et al., 2006a; Jayamani et al., 2007). Four alleles of this locus indicated as $(CT)_{16}$, $(CT)_{17}$, $(CT)_{18}$ and $(CT)_{19}$ respectively were also detected in 56 of waxy rices and class $(CT)_{19}$ is associated with high gelatilization temperature (Bao et al., 2002b). The G to T SNP and CT SSR have already been utilized in marker-assisted selections (Bergman et al., 2001; Ramalingam et al., 2002; Zhang et al., 2005; Jayamani et al., 2007). Four other SNPs were found in the coding sequence of *Wx* gene that resulted in amino acid substitutions (Sato et al., 2002; Larkin and Park, 2003). These SNPs are associated with variations in amylose content and viscosity characteristics. Several other polymorphisms in starch synthetase genes have been described that are associated with physicochemical properties of grain, such as SSRs in SBE1 and SSSI, SNPs in SBE3 and SSIIa, a sequence tagged site (STS) in SBE1 and one insertion/deletion (InDel) in SSIIa (Bao et al., 2002b; Umemoto et al., 2004; Bao et al., 2006b, a). Compared to the research on starch synthetases, there are only few studies on genetic diversity of seed-storage protein genes in rice. In the glutelin gene *GluD-1*, a total of 28 SNPs were detected in the sequences of four japonica and indica cultivars (Kawakatsu et al., 2008). However these SNPs have not yet been reported to be associated to any variation of seed-storage protein content in rice.

Identification of important genes affecting different quality traits by mapping based approaches

Dwarfism in plants is long known to be an attractive characteristic in crop breeding. Although the most obvious phenotypes of dwarf rice are reduced plant height and erect leaves, many mutants also bear small and round grains. However, the molecular bases of such phenotypes have not been well elucidated till the rapid development of genetic approaches such as map-based cloning in the last two decades. *Dwarf-1 (D1)* gene has been mapped on the chromosome 5 as the α-subunit of GTP-binding protein (G protein) (Cho et al., 1994; Fujisawa et al., 1999). The *d1* mutant was insensitive to both gibberellins (GA) (Mitsunaga et al., 1994; Ueguchi-Tanaka et al., 2000; Bethke et al., 2006) and brassinosteroid (BR) (Wang et al., 2006; Oki et al., 2009), suggesting that G protein is essential for both GA and BR pathways. The cell number in *d1* leaf sheath, the internode, the root and the lemma was reduced compared to the wild type (Izawa et al., 2010), which might also be the reason for the small seeds in the mutant. Studies of *d61* (Yamamuro et al., 2000), *d2* (Hong et al., 2003), *d11* (Tanabe et al., 2005) and *brd2* (Hong et al., 2005) dwarf mutants have identified *OsBR1* encoding a putative BR receptor kinase, *CYP90D2, CYP724BI*, and *DIM/dwf1* encoding three BR

biosynthesis enzymes, as the molecular bases of dwarfism in those mutants. The four genes were mapped on chromosome 1, 1, 4 and 10 respectively. Besides the dwarf mutants, another *srs3* mutant exhibited small and round seeds as well as shortened panicles and internodes. It was caused by the defect in a novel kinesin 13 protein gene *SRS3* which was mapped on chromosome 5. The cell length of seeds in the longitudinal direction in *srs3* is shorter than that in the wild type which results in the reduced length of the grains (Kitagawa et al., 2010).

Searching of QTLs revealed that many traits of grain quality are not controlled by single genes but involve multiple regions in the rice genome. In doubled haploid and other inbred lines, two major loci corresponding to the *Wx* gene and the alkali degeneration gene (*alk*) gene on chromosome 6, were identified as QTLs responsible for the main variance of amylose content and gelatinization temperature traits respectively (He et al., 1999; Tan, 1999; Li et al., 2003; Bao et al., 2004b; Fan et al., 2005; Takeuchi et al., 2007). The *Wx* locus also showed a major effect on the property of gel consistency (Fan et al., 2005). These further substantiate the important roles of the two genes and the regions tightly linked to these loci in determining the cooking quality of rice. In addition, various QTL studies have identified three other major genes, *GS3* for grain length (Huang et al., 1997; Redoña and Mackill, 1998; Tan et al., 2000; Kubo et al., 2001; Thomson et al., 2003; Aluko et al., 2004; Fan et al., 2006), *GW2* (Song et al., 2007; Guo et al., 2009) and *GW5 (qSW5)* (Shomura et al., 2008a; Weng et al., 2008) for grain width, which are localized on chromosome 3, 2 and 5 respectively. *GS3* encodes a protein of 232 amino acids with a putative PEBP-like domain, a trans-membrane region, a putative TNFR/NGFR family cysteine-rich domain and a VWFC module. *GW2* encodes a RING-type protein with E3 ubiquitin ligase activity and *GW5* encodes a novel protein without significant homology to any proteins of known biochemical functions. Besides, QTLs responsible for minor variations in traits like appearance, eating and cooking with minor effects have been mapped on different other loci (Huang et al., 1997; Redoña and Mackill, 1998; Bao et al., 2000; Tan et al., 2000; Bao et al., 2002a; Aluko et al., 2004; Wan et al., 2004; Tanaka et al., 2006; Wada et al., 2006; Yoon et al., 2006; Tabata et al., 2007; Takeuchi et al., 2007; Kobayashi and Tomita, 2008; Kobayashi et al., 2008; Zhang et al., 2008a; Sabouri, 2009).

Effects of genes involved in flower development is mainly on grain appearance

Caryopses in cereals such as corn, wheat and rice develop from the fertilized pistil, in which the ovary wall is united with the seed coat, making it difficult to separate and unique from other plants. The early development of floret organs has indirect but profound influences on seed development and thus affects the quality and yield of the grains. The rice panicle or inflorescence is of the compound raceme type which consists of a rachis which is the main axis, the primary rachis branches and the secondary rachis branches. The pedicels grow from each node on the secondary branch and the tip of the primary branch. Spikelets are attached to the top of the pedicels. The numbers of rachis and spikelet differ among cultivars and the yielding capacity is decided by the ability to form more secondary branches and spikelets (Yamamoto et al., 1991; Park et al., 1999; Yoshida et al., 2006). Based on the latest results, development of panicles and spikelets goes through nine and eight stages respectively with specific landmark events (Ikeda et al., 2004; Itoh et al., 2005). Rice is highly self-pollinated. Each spikelet contains a single floret and two pairs sterile glumes which are the rudimentary glumes and the empty glumes. The floret comprises of a lemma, palea and three kinds of organs: two lodicules (petals), six stamens and one pistil constituted by a single

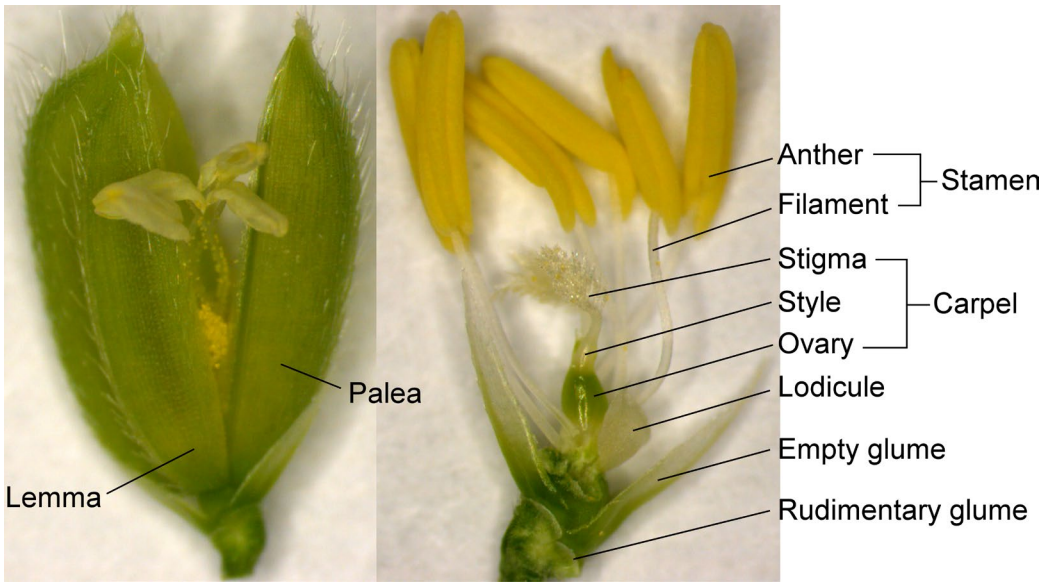

Figure 2. Structure of a rice flower

carpel (Figure 2). The florets open either in the morning or in the afternoon dependant on the cultivar used and weather conditions (DeDatta, 1981). Anthesis in rice is started from the movement of the lemma away from palea till the flowers are widest open; after that, the lemma is moving back towards the palea and finally the floret closes again (Chapter 3, this thesis). The filaments elongate during flowering and anthers of the stamens emerge between the dehisced palea and lemma. The tips of the anthers remain outside the floret even when the flowering process is completely finished. The pistil also appears at the base of opened lemma and palea during flowering but it is enclosed again in the spikelet after floret closure (Matsuo and Hoshikawa, 1993).

Because rice grain is developed from flowers, the role of floret development on grain quality is also essential. However, the effects are indirect which makes the research in such area difficult. Functions of genes from several different families, involved in inflorescence architecture as well as in grain characteristics, have been described recently. Overexpression of the Arabidopsis jasmonic acid carboxyl methyltransferase gene (*AtJMT*) in rice increased levels of methyl jasmonate (MeJA) by six-fold in young panicles. Transgenic plants had altered numbers of spikelet organs, including the lemma/palea, lodicule, anther, and pistil. However, grain yield, number of spikelets per panicle, and filling rate were largely reduced (Kim et al., 2009). Two different genes controlling the panicle erectness have been identified in japonica rice (Huang et al., 2009; Zhou et al., 2009) as well as in indica rice (Zhu et al., 2010) respectively. The *DEP1* (*qPE9-1*) gene encodes a protein homologous to the keratin-associated protein 5-4 family and EP2 is a protein localizes to the endoplasmic reticulum with unknown function. Loss of function of both genes results in an erect panicle phenotype. In addition, they also regulate other panicle characteristics such as panicle length, grain size and number. The putative nitrate transporter SP1 was identified in a rice mutant, *short panicle1* (*sp1*), which is defective in panicle elongation resulting in a short-panicle phenotype with reduced seed size and seed number per panicle (Li et al., 2009). A chromosome segment substitution line CSSL58, developed from backcross progenies (BC3F2) derived from a cross between a indica

cultivar Teqing (*O. sativa* L.) as the recurrent parent and wild rice (*O. rufipogon* Griff.) as the donor parent, contains a chromosome segment of *O. rufipogon* introgressed in the genetic background of the indica cultivar and showed significantly smaller panicles, fewer grains per panicle, smaller grains and dwarfness. Phenotypic segregation revealed that these traits are controlled by a recessive gene *spd6* from wild rice and fine mapping narrowed down *spd6* to a 22.4 kb region on chromosome 6. Four candidate genes are located within this segment (Shan et al., 2009). The major QTL *qSW5*, which is involved in determination of grain width in rice, controls the number of rows of specialized cells with rigidified walls in the upper epidermis and especially of the outer glume (lemma) of the flower. This indicates that the primary cause of the increase in grain width is the increase in size of the outer glumes (Shomura et al., 2008b). The MADS-box protein FRUITFULL (FUL) was first shown to be required for carpel and fruit development in Arabidopsis (Gu et al., 1998). Over-expressing two members of the microRNA family (miR172) in rice resulted in delayed transition from spikelet meristem to floral meristem, and resulted in floral and seed developmental defects, including changes to the number and identity of floral organs, lower fertility and abnormal appearing seeds (Zhu et al., 2009). In rice, the *FUL* like genes identified so far include *OsMADS14*, *OsMADS15* and *OsMADS18*. However, for none of them a function was demonstrated in development of seeds.

Effects of the environment on different quality traits

Effects of water-shortage and drought on grain quality

Water is one of the most essential inputs for production of crops and rice among all the crops consumes the most water for irrigation. In many systems production of 1 kilogram rice takes 5,000 liter of water although this can be reduced to about 2,000 (Lal, 2007). The water status of the soil has a dramatic effect on yield and it also affects the quality of the grain (Dingkuhn and Le Gal, 1996). Traditionally rice is planted under submerged condition, however non-flooded plastic film mulching (PM) and non-flooded wheat straw mulching (SM) have been considered as new water-saving techniques in rice production. Different water management treatments, namely PM, water-saving irrigations and conventional irrigations, significantly affected brown rice rate, head rice rate, chalky grain rate, amylose content and protein content in a cultivar and grain position dependent manner. Of all variables, water treatment had the strongest effect on protein content (Cheng et al., 2003b). SM was found to significantly increasing milling quality and reduced the percentage of chalky grain, chalky size and chalkiness while PM showed opposite effects. Gel consistency was found decreased under PM (Zhang et al., 2008b). Rice is the only crop which can survive periods of submergence. However, flooding just before harvest brought visible changes to the physical appearance of grains. The kernels in flood-affected samples became soft and developed fissures which contributed to low head rice recoveries and the milled rice had lower kernel weight and protein content but showed higher amylose and ash content (Singh et al., 1990).

Upland non-flooded environment also affects grain quality compared to the lowland condition. A recent study compared several cooking and nutrient quality traits, including the amylose content, the gel consistency, the gelatinization temperature, and the protein content, in the same populations grown under upland and lowland conditions. The phenotypic values of all four traits were significantly higher under upland environment than lowland environment (Guo et al., 2007).

Fertilizer application

Nitrogen level in the soil strongly affects yield and quality of the grain. It was hypothesized that the yield is related to the nitrogen supplying capacity of soil, which determines the grain protein content (Perez et al., 1996). Application of nitrogen fertilizer at different stages from panicle initiation, heading, flowering to grain filling has all been shown to strongly increase seed-storage protein content for a long time (Nangju and De Datta, 1970; Taira, 1970; Seetanun and De Datta, 1973; Nagarajah et al., 1975; Vaughan et al., 1980; Perez et al., 1990; Perez et al., 1996; Souza et al., 1999; Leesawatwong et al., 2004, 2005) as well as protein related traits like the milled rice rate, head rice rate (Wopereis-Pura et al., 2002; Leesawatwong et al., 2005) and translucency (Perez et al., 1990, 1996). In addition, it was also reported that application of nitrogen decreased the amylose content of rice kernel, while this treatment did not significantly affect the protein content (Bahmaniar and Ranjbar, 2007). Besides nitrogen, potassium is another essential fertilizing element for rice production (Fageria et al., 1990a, 1990b). The effects of potassium on grain quality were not well understood till a recent discovery showing that application of potassium fertilizer increased gel consistency and grain protein content but had no significant effect on gelatinization temperature and amylose content (Bahmaniar and Ranjbar, 2007).

Salinity of the soil

Salinity is another important condition of the soil which affects quality of the grain. A comparison of rice cultivars grown in low salinity and high salinity showed that an increased level of salinity significantly lowered the protein content in seven of nine varieties, but had no effect on amylose content and the alkali spreading value (Juliano and El-Shirbeeny, 1981). Another study indicated that grains of both saline tolerant and susceptible varieties grown on saline soils have higher storage protein content, but less translucent grain, lower starch and amylose content than grown on normal soil. Thus these differences were not related to salinity tolerance (Siscar-Lee et al., 1990).

Season and temperature

Rice is mostly grown as a transplanted crop. The delay of sowing and transplanting date affects the grain quality due to the change of temperature and solar radiation. Late planting of the rice delays the flowering and results in partial filling of the spikelets, thus lowering the milling yield and head rice recovery during processing (Shahi et al., 1975; Dhaliwal et al., 1986). The grain dimensions (length/width) were affected by late sowing and transplanting. Late transplanting resulted in higher protein content values as well. However, the amylose content decreased due to late sowing and transplanting (Dhaliwal et al., 1986). The reduction of amylose may be to some extent owing to the higher temperature during grain filling because of the late sowing and transplanting. It was reported that *Wx* gene expression was increased in response to low temperature (18°C). The longer rice plants were exposed to low temperature, the higher the levels of Wx protein and the greater the accumulation of amylose (Hirano and Sano, 1998). The G-T polymorphism within the first intron of *Wx* gene has been reported to be related to the different efficiency of RNA splicing and processing when treated with low and high temperatures during the period of grain development (Larkin and Park, 1999). This could be one of the crucial reasons behind the different level of *Wx* transcripts under different temperatures. Besides *Wx* transcripts, starch branching enzymes especially BEIIb were reported down-regulated by high temperature, whereas α-amylases were up-regulated

(Yamakawa et al., 2007). Apart from the effects on transcription, the regulation of temperature is also reflected by its influences on the activities of key enzymes for the biosynthesis of starch (Cheng et al., 2003a; Jiang et al., 2003; Satoh et al., 2008). Thus the structure and composition of amylose and amylopectin are affected by temperature. For instance, temperature change during grain-filling affects the chain length distribution of amylopectin (Umemoto et al., 1999; Yamakawa et al., 2007). High temperature also causes reduction in the amount of large mature amyloplasts and increase in the number of small immature amyloplasts containing single starch granule (Zakaria et al., 2002). Besides starch, storage protein content is also affected by temperature. Elevated temperature decreases accumulation of prolamin, which is consistent with diminished expression of prolamin genes (Yamakawa et al., 2007; Ma et al., 2009). On the other hand, the relative content of glutelin is not changed with high temperature stress, but the relative content of two glutelin subunits was reduced and the amount of glutelin precursor was improved (Ma et al., 2009). High temperature stress results in a severe chalky appearance which is due to the change of starch and protein composition. Recently, a study on gene expression in combination with metabolite measurements in rice endosperm revealed several possible key steps for the inhibition of starch accumulation and the accumulation of amino acids in the developing caryopsis exposed to high temperature. It provided us more comprehensive knowledge of the mechanism behind high temperature stress on determination of the grain quality (Yamakawa and Hakata, 2010).

Hormonal regulation

Ethylene is a plant hormone regulating fruit ripening by coordinating the expression of genes that are responsible for a variety of processes, including an increase in respiration, autocatalytic ethylene production and changes in color, texture, aroma and flavor. In rice, ethylene evolution rate was significant and negatively correlated with grain-filling rate (Liu et al., 2008), resulting in an inverse correlation with chalky kernel percentage and chalkiness (Yang et al., 2007; Zhang et al., 2009a). Cultivars with a low 1-aminocylopropane-1-carboxylic acid (ACC) concentration in grains exhibited a close amyloplast arrangement and little space between starch granules, whereas those with a high ACC concentration in grains showed a loose arrangement and wide space between the granules (Yang et al., 2007). Application of ACC to panicles at early (Zhang et al., 2009a), mid and late (Yang et al., 2007) grain filling stages significantly increased chalky kernel percentage, chalky area and chalkiness. The results were reversed when amino-ethoxyvinylglycine, an inhibitor of ACC synthesis enzyme, was applied to panicles. These effects of ethylene on appearance of the grain may largely relate to the activities of the key enzymes involved in sucrose to starch conversion in the grains. ADP glucose pyrophosphorylase and sucrose synthase activities were found to be negatively correlated with ethylene concentration (Mohapatra et al., 2009). Application of amino-ethoxyvinylglycine to panicles at the early grain filling stage significantly increased activities of sucrose synthase, ADP glucose pyrophosphorylase and soluble starch synthase (Zhang et al., 2009b). In addition, the effects of ethylene depend on the positions of spikelets on the panicles, with stronger influences on grain filling and quality of the kernels of the basal spikelets (Naik and Mohapatra, 2000). Furthermore, several studies revealed that the interaction between abscisic acid (ABA) and ethylene may be involved in mediating the post-anthesis development of spikelets (Yang et al., 2004, 2006; Zhang et al., 2009b). Application of gibberellin and cytokinin have been also reported to enhance development of spikelets, while indole-3-acetic acid (IAA) showed a depression compared

to the controls (Patel and Mohapatra, 1992). Alteration of ethylene, ABA and gibberellin levels in rice is often related to water deficit and drought stress which affects the grain-filling rate and eventually changes the yield and quality of the grains (Yang et al., 2001,, 2004; Liu et al., 2008; Zhang et al., 2009b).

Conclusions

In summary, grain quality in rice is determined by a variety of internal developmental processes which are affected by environmental conditions as well as the genetic constitution. Mutations of genes encoding starch synthetases and storage glutelin alter the composition of the endosperm, thus affect the appearance, cooking and nutritional quality. Meanwhile, regulatory proteins play important roles both on transcription and post-transcription levels. Besides that, studies on genetic diversity revealed the associations of certain SNP and SSR markers in starch synthetase genes to the physicochemical properties of rice grain. In recent years, mapping-based cloning and identification of QTLs resulted in the identification of several important genes associated to different quality traits, especially the size of the kernel. In addition, several genes involved in the development of flowers show their profound influences on grain quality.

On the other hand, the effects of the environment cannot be ignored. Water and drought stress alter the ratio of ethylene, ABA and gibberellin in seeds in turn affecting the grain filling rate and milling quality of the grain. Nitrogen and potassium fertilizer application increases the content of seed-storage protein. Gene expression and enzyme activity of some starch synthethases may vary at different temperatures. Elevated temperature also influences the protein composition of the grain. Nevertheless, the effects of the environment are to a certain extent dependent on the genetic constitution. Significant genotype × environment effects were found in the statistical analysis of different quality traits from rice cultivars grown in different locations and seasons (Bao et al., 2004a; Cameron et al., 2008; Sharifi et al., 2009). These further substantiate the key roles of genetic factors in determination of grain quality in rice.

Thesis outline

Grain quality is second to yield as the major breeding objective in rice breeding. Much progress has been made in the last two decades in understanding the molecular basis behind different quality traits. The aim of this research was to identify novel genes important for the development of seeds and flowers, which in turn will affect quality traits of the rice grain.

In **Chapter 2** we used a yeast one-hybrid approach to screen for novel transcription factors for storage glutelin gene *GluB-1*. cDNA libraries of rice panicle and seed were constructed and screened with the core promoter of *GluB-1* as bait sequence. Two CCCH zinc finger proteins, OsGZF1 and OsGZF2, have been discovered binding specifically to the bait in yeast, which was confirmed by EMSA afterwards. Northern blot, RNA *in situ* hybridization and promoter-GUS transgenic rice were used to study the spatial and temporal expression of *OsGZF1* and *OsGZF2*. Transient assays verified their repression function on *GluB-1* promoter. We also analyzed the influences of the two proteins on a strong *GluB-1* activator RISBZ1 *in vivo*. *OsGZF1* and *OsGZF2* overexpressors, mutants and RNAi plants were used to further confirm their biological function *in vivo*.

Chapter 3 and **Chapter 4** focused on genes involved in flower development because structure and development of flowers might also have potential and profound roles in grain quality determination. In **Chapter 3** a collection of T-DNA and transposon mutants ordered from different collections were studied. In two *Tos17* transposon insertion lines, *osjar1-2* and *osjar1-3*, seeds with abnormal color and shape were observed. Southern blot analysis revealed that the T-DNA insertions were in the exons of a GH3 family gene *OsJAR1*. Knock-out of the gene was confirmed by northern blot analysis. Besides the seeds, phenotypical differences were mainly visible in flowers. *osjar1* flowers failed to close after dehiscence, which could be rescued by overexpressing *OsJAR1* in the mutant background. To identify the biological function of OsJAR1, the gene expression pattern was analyzed first. In a follow-up approach using *in vitro* enzymatic reactions with recombinant OsJAR1 protein and *in vivo* measurements on wild type and *osjar1* extracts demonstrated that OsJAR1 acts as a jasmonic acid-amino acid (JA-AA) synthetase in rice.

OsJAR1 was confirmed to conjugating JA to AA, yet through which pathway JA-AA regulates flower opening and closure is unsolved. We speculate that the reason why *osjar1* flower failed to close after flowering is probably due to the abnormal K^+ accumulation in lodicules which disturbs the change of water potential during floret closure. In **Chapter 4,** *osjar1* lodicules were indeed found to contain twice the amount of K^+ compared to the wild type. Candidate transporter genes were selected from literature and databases and further analyzed. A monovalent cation/proton anitporter-2 family gene *OsCHX14* was the only one that showed an expression polymorphism between flowers from wild type and *osjar1*. Histochemical staining of ProOsCHX14-GUS transgenic plants showed the gene is predominantly expressed in the lodicules. Subcellular localization of the protein was analyzed in rice protoplasts. Yeast complementation assays revealed that OsCHX14 was able to efflux K^+ outside of the cells. These results support a function for OsCHX14 in regulation of floret closure after flowering by effluxing K^+ from the lodicule cells.

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