

Knockout of a starch synthase gene *OsSSIIIa/Flo5* causes white-core floury endosperm in rice (*Oryza sativa* L.)

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Received: 3 December 2006 / Revised: 12 January 2007 / Accepted: 15 January 2007 / Published online: 13 February 2007
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Abstract To elucidate the role of SSIIIa during starch synthesis in rice (*Oryza sativa* L.) endosperm, we characterized null mutants of this gene, generated by T-DNA insertions. Scanning electron microscope (SEM) analysis revealed that the starch granules in these mutants are smaller and rounder compared with the wild type controls, and that the mutant endosperm is characterized by a loosely packed central portion exhibiting a floury-like phenotype. Hence, the *OsSSIIIa* (*Oryza sativa* SSIIIa) mutations are referred to as *white-core floury endosperm 5-1* (*flo5-1*) and *flo5-2*. Based upon their X-ray diffraction patterns, the crystallinity of the starch in the *flo5* mutant endosperm is decreased compared with wild type. Through determination of the chain-length distribution of the mutant

endosperm starch, we found that *flo5-1* and *flo5-2* mutants have reduced the content of long chains with degree of polymerization (DP) 30 or greater compared with the controls. This suggests that *OsSSIIIa/Flo5* plays an important role in generating relatively long chains in rice endosperm. In addition, DP 6 to 8 and DP 16 to 20 appeared to be reduced in endosperm starch of *flo5-1* and *flo5-2*, whereas DP 9 to 15 and DP 22 to 29 were increased in these mutants. By the use of differential scanning calorimetry (DSC), the gelatinization temperatures of endosperm starch were found to be 1–5°C lower than those of the control. We propose a distinct role for *OsSSIIIa/Flo5* and the coordinated action of other SS isoforms during starch synthesis in the seed endosperm of rice.

Communicated by J.R. Liu.

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Keywords *Floury endosperm* · Flo5 · OsSSIIa · Rice · Starch synthase

Introduction

Starch is the major storage polysaccharide in many plants, including rice, maize, and potato. Stored starch generally consists of two D-glucose homopolymers, amylose and amylopectin. Amylose is a linear polymer composed of 1,4-linked α -D-glucopyranosyl residues. Amylopectin, a more abundant polymer in starch, is a highly branched glucan with α -1,6 glucosidic bonds which connect linear chains (Nakamura 2002; James et al. 2003). The structure of amylopectin contributes to the crystalline organization of the starch granule (Buléon et al. 1998; Nakamura 2002) such that fine amylopectin clusters are responsible for the properly formed endosperm starch that comprises the largest portion of the seeds (Kubo et al. 1999; Nishi et al. 2001; Burton et al. 2002; Fujita et al. 2003; Satoh et al. 2003). Starch synthesis in the endosperm of cereals requires a concerted series of enzymatic reactions involving ADP glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme (DBE). SS elongates linear glucan chains by catalyzing the transfer of a glucosyl unit of ADP glucose (ADPGlc) to the non-reducing end of a glucan chain. BE is a unique enzyme that can catalyze the formation of α -1,6 glucosidic linkage in a polyglucan chain. On the other hand, DBE is known to hydrolyze α -1,6 glucosidic linkages of polyglucan.

In higher plants, five types of SS, one granule-bound SS (GBSS) and four soluble SSs, have been classified on the basis of their deduced amino acid sequences (Hirose and Terao 2004; Ohdan et al. 2005). GBSS, the first of these classes, is responsible for the elongation of amylose chains that carry no branches. Of two identified GBSS isoforms, the role of GBSSI is mostly confined to storage tissues, whereas GBSSII is involved in amylose synthesis in non-storage tissues in which transitory starch is accumulated (Vrinten and Nakamura 2000; Dian et al. 2003; Hirose and Terao 2004). Many studies have found that higher plants have four distinct types of soluble SSs (SSI to SSIV) and each has multiple isoforms. It has been speculated that rice has eight isoforms of soluble SS: an SSI (OsSSI), three SSII (OsSSIIa, OsSSIIb and OsSSIIc), two SSIII (OsSSIIIa and OsSSIIIb), and two SSIV isoforms (OsSSIVa and OsSSIVb). Most of the rice SS isoforms are found in the endosperm (Hirose and Terao 2004; Jiang et al. 2004; Dian et al. 2005).

On the basis of their gene expression patterns in developing seeds, soluble SSs are grouped into three classes in rice: early, late, and steady expressers (Hirose and Terao 2004). The developmental processes in the rice seed endosperm consist of a pre-storage and a starch filling phase. *OsSSIIb* and *OsSSIIIb* belong to the early expressers and are predicted to function predominantly at the pre-storage phase of seed endosperm development—1–5 days after fertilization (DAF)—during which their expression is mainly observed. Late expressers include *OsSSIIa* and *OsSSIIIa*, whose transcripts are rarely detectable during the pre-storage stage but are abundant at the starch filling phase after the five DAF stage (Hirose and Terao 2004; Ohdan et al. 2005). This group is believed to play a critical role during starch synthesis in the seed endosperm. The expression of the remaining four genes, *OsSSI*, *OsSSIIc*, *OsSSIVa* and *OsSSIVb*, is relatively constant during endosperm development. These data suggest that each SS isoform has a distinct role in starch synthesis in the developing rice endosperm.

Among the rice soluble SSs, the roles of some isoforms have been characterized either by analyzing their loss-of-function mutants and gain-of-function transgenic plants, or by examining the relationship between SS activity and starch structure in a number of different cultivars. The function of the rice SSI has been recently investigated in the *Tos17* insertional mutant lines (Fujita et al. 2006). The result indicated that this enzyme plays a critical role in generating chains with degree of polymerization (DP) 8 to 12 from the short DP 6 to 7 that emerge from the branch point of amylopectin. Since SSI mutations do not affect the size and shape of seeds and starch granules, nor the crystallinity of endosperm starch, it is probable that other SS enzymes, in part, compensate for the loss of SSI function. *OsSSIIa* is known to correspond to the *alk* (alkali disintegration)/*gel* (gelatinization) gene, which causes the differences in the disintegration of endosperm starch granules in cases of alkali steeping and their gelatinization between *japonica* and *indica* rice varieties (Umemoto et al. 2002; Nakamura et al. 2005). These previous studies have suggested that *OsSSIIa* preferentially elongates DP 6 to 11 to DP 13 to 28, and that the activity of *OsSSIIa* in *japonica* rice is reduced, relative to its activity in *indica* rice. The other isoforms of this enzyme remain to be studied in related mutants to further clarify their precise role in starch synthesis.

The gene expression pattern of *OsSSIIIa* suggests that it plays a role during the starch filling phase of the developing endosperm (Hirose and Terao 2004; Dian et al. 2005; Ohdan et al. 2005). Previously reported analyses of *SSIII* mutants, such as the maize *dull-1* (Gao

et al. 1998; Jane et al. 1999) and *Arabidopsis Atss3-1* and *Atss3-2* (Zhang et al. 2005), and antisense-*SSIII* potato (Edwards et al. 1999; Lloyd et al. 1999), have indicated that in these plants, SSIII produces the relatively long chains of amylopectin B₂ (DP 25 to 36 which carries one or more chains belonging to only one cluster) and B₃ to B₄ (≥DP 37; Hanashiro et al. 1996). A purified OsSSIIIa fraction in rice endosperm appears also to generate long chains from chains with ≤DP 11 in vitro when glycogen is used as the primer (Fujita et al. 2006).

Studies of opaque endosperm mutants, such as dull, floury, and glutinous endosperms, have often proved to be useful in identifying the genes encoding starch biosynthetic enzymes. For example, the investigation of the *amylose-extender (ae)* mutant lacking a starch branching enzyme BEIIb, facilitated our understanding of the specific enzymatic role in the transfer of short chains of amylopectin in the endosperm (Nishi et al. 2001). Some *floury endosperm (flo)* phenotypes in rice have also been found to be involved in starch granule morphologies. The *flo1* locus is known to generate a floury-white endosperm that contains round and loosely packed starch granules (Satoh and Omura 1981). *Flo2* is a mutant with endosperm of a high lysine content and increased level of histidine (Kumamaru et al. 1997). This mutation was also found to regulate the expression of the starch synthesis genes (Kawasaki et al. 1996). Recently, *flo4*, a rice mutant with an abnormal endosperm consisting of loosely packed starch granules, appeared to harbor a mutation in the *OsPPDKB* gene encoding pyruvate orthophosphate dikinase (PPDK) (Kang et al. 2005).

In the present study, we analyzed two rice *SSIIIa* mutants that we generated by T-DNA insertions in the coding sequence of the gene. Both mutant alleles resulted in a white-core floury endosperm, and are thus designated as *flo5-1* and *flo5-2*. Examination of the chain-length distribution of the amylopectin and physicochemical traits of starch in the endosperms of these mutants further revealed a distinct role of OsSSIIIa/Flo5 during amylopectin biosynthesis in the developing endosperm of rice. Finally, we discuss the evidence that amylopectin synthesis is mediated by the coordinated functions of the SS isoforms, SSI, SSIIa and SSIIIa/Flo5, in rice endosperm.

Materials and methods

Plant materials

Two *japonica* rice (*Oryza sativa* L.) cultivars Dongjin and Hwayoung and their respective mutants, *flo4-1*

and *flo4-2*, and *flo5-1* and *flo5-2*, were grown during summer months under natural environmental conditions in an experimental field of Kyung Hee University. For the preparation of starch samples, mature seeds were harvested and stored at −20°C. The ripening grains were collected at the late milking stage, frozen in liquid nitrogen and kept at −80°C until needed.

Isolation of *OsSSIIIa/Flo5* mutants

Two alleles harboring *OsSSIIIa* mutations, *flo5-1* and *flo5-2*, were identified from a screen of the rice T-DNA Insertion Sequence Database (RISD; Jeon et al. 2000; Jeong et al. 2002; An et al. 2003; Jeong et al. 2006; <http://www.141.223.132.44/pfg/index.php>). Both pGA2715 and pGA2772, that have been used to generate rice mutants, are activation tagging vectors that contain a 35S enhancer element in their T-DNAs. pGA2772 is a modified version of pGA2715 and contains the pUC18 vector backbone. This vector is thus useful for retrieving T-DNA flanking regions (Jeong et al. 2002, 2006). Homozygous mutants of segregating individuals were isolated by PCR screening using a T-DNA-specific primer T1 5'-ATCCA GACTGAATGCCACA-3', and the *OsSSIIIa* gene-specific primers, F1 5'-TGAAAACCTTCCAAGTCC AAAATCAGT-3'; R1 5'-GCATCTGACATAGGA TGAAATAAGCAAAA-3'; F2 5'-GTTTTGATTCA TTTCATCTTGGGAACATA-3'; and R2 5'-TTTAC GAAGCTATCCTACACAAACCTGAA-3' (for the location of these primers, see Fig. 1a).

RT-PCR analysis

Total RNAs were prepared from ripening grains using Trizol reagent (Invitrogen, MD, USA; <http://www.invitrogen.com>). DNase-treated RNA preparations were reverse-transcribed with an oligo-dT primer and a first strand cDNA Synthesis kit for RT-PCR (Roche, Penzberg, Germany; <http://www.roche-applied-science.com>). First-strand cDNA was used in these PCR reactions with gene-specific primers and control primers for *OsAct1* (McElroy et al. 1990; Cho et al. 2006). Gene-specific primers were designed to encompass the 3rd and 4th introns and exclude possible genomic DNA contamination (for the location of primers, see Fig. 1a). The primers were: F3 5'-CATGA AGTTGATGTAATCTCTTTG-3' and R3 5'-TCTCA TAGTCTTTTCCCTTCATCTC-3'; and *OsAct1* 5'-GG AACTGGATAGGTCAAGGC-3' and 5'-AGTCTCA TGGATACCCGCAG-3'. The amplification program

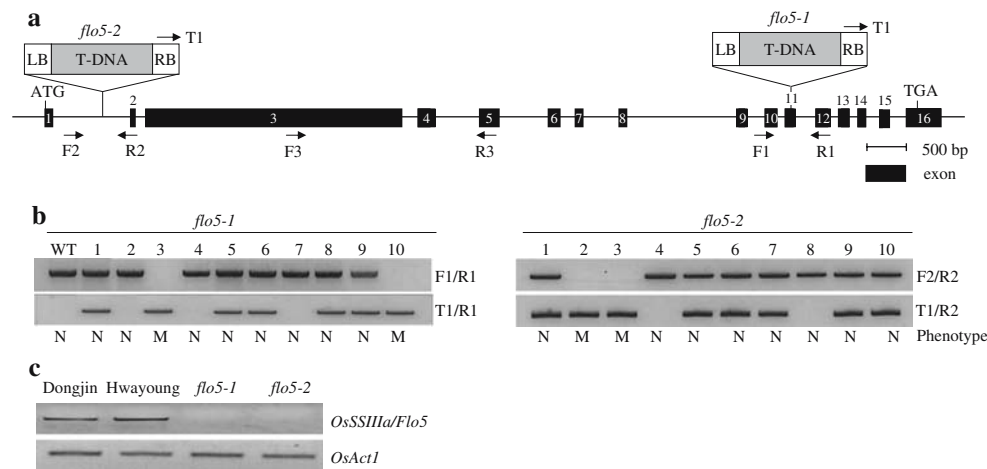


Fig. 1 **a** Position of the T-DNA insertions in two *flo5* mutant alleles. The T-DNA insertion positions in *flo5-1* and *flo5-2* are shown schematically in a diagram of the *OsSSIIIa* genomic structure. Numbers in the squares signify exons. A T-DNA is inserted in the 11th exon in *flo5-1*, and in the first intron in *flo5-2*. Primers used in the genotype analysis are indicated by arrows. F1/R1 and F2/R2 are gene specific primer sets, and T1 is -DNA-specific primer. Primers F3 and R3 were designed for RT-PCR analysis and encompass a portion of exon 3, intron 4, and exon 4. The translation start site and stop codons are indicated by ATG and TGA, respectively. **b** Genotypic and phenotypic analysis of segregating T2 progeny of the *flo5* mutants. PCR analyses using

two sets of primers (F1/R1 and T1/R1 for *flo5-1* and F2/R2 and T1/R2 for *flo5-2*) and genomic DNA as the template indicate that two individual plants (#3, #10) in *flo5-1*, and 2 plants (#2 and #3) in *flo5-2* are homozygous for the T-DNA insertions. The homozygous plants exhibit a mutant (M) phenotype and the others including wild type (WT) are normal (N). **c** *OsSSIIIa/Flo5* gene expression in developing seed endosperms of wild type plants, Dongjin and Hwayoung, and *flo5* mutants. RT-PCR analysis using F3 and R3 primers indicates that the *flo5* mutants lack the endogenous gene. Transcript for the rice actin gene *OsAct1* was amplified as RT-PCR control

consisted of an initial 94°C for 5 min, followed by 28–35 cycles of 94°C, 1 min; 56°C, 1 min; 72°C, 1 min, and a final extension of 72°C for 5 min. Each amplification was repeated three times.

Measurement of starch contents

Starch contents were measured in the insoluble fractions of ethanol–water extracts (Lee et al. 2005), and determined spectrophotometrically as described previously (Jelitto et al. 1992).

Preparation of insoluble starch from rice endosperm

Starch was purified from polished seeds of homozygous mutant lines and their respective wild types using an alkali steeping isolation method. Briefly, rice flours were steeped repeatedly in 0.1% aqueous NaOH solution and washed with sterilized water (Patindol and Wang 2003). The isolated starch was then dried at room temperature, ground into powder and then passed through 100 mesh sieve. The isolated starch powders were then analyzed by microscopic observation,

X-ray diffraction pattern, chain profiles of amylopectin and thermal properties.

Microscopic analysis

To detect the floury endosperm phenotype, husked rice seeds were observed on an illuminator. Scanning Electron Microscope (SEM) analysis was carried out according to Fujita et al. (2003). Starch samples were coated with gold and the mounted specimens were observed under a Stereoscan Leica Model 440 instrument (Leica Cambridge, UK; <http://www.leica-microsystems.com>) at an accelerating voltage of 20 kV.

X-ray diffraction measurement

X-ray diffraction patterns were obtained using an X-ray diffractometer (M18XHF, Mac Science Co., Japan; <http://www.macscience.co.jp>), operating at 40 kV and 300 mA and producing Cu-K α radiation at a wavelength of about 1.542 Å. Starch was scanned from 4° to 40° of the diffraction angle 2θ with a scanning rate of 3°/min (0.02° step). The measurements were performed at ambient temperature. The relative crystallinity of starch was quantitatively estimated by the method of Komiya and Nara (1986).

Chain-length distribution of amylopectin

The chain-length distribution of starch extracted from mature endosperm was determined according to the previously described high-performance anion exchange chromatography (HPAEC) method (Kubo et al. 1999; Kang et al. 2003). To determine the distribution of chain length by HPAEC-PAD (HPAEC equipped with a pulsed amperometric detector), starch (1 mg/ml) was suspended in distilled water, and then boiled for 60 min. One milliliter of this gelatinized sample was then added to 50 μ l of 600 mM sodium acetate buffer (pH 4.4) and 10 μ l of 2% (w/v) NaN_3 , and hydrolyzed by the addition of 700 U of isoamylase (I2758, Sigma, St. Louis, MO, USA; <http://www.sigmaaldrich.com>) at 37°C for 24 h. The debranched glucans were then reduced with sodium borohydride under alkaline pH conditions for 20 h and the precipitate was dried at room temperature. The reduced isoamylolysate sample was next dissolved in 50 μ l of 1 M NaOH for 60 min and diluted with 450 μ l of distilled water. A 25- μ l aliquot of this preparation was injected into a BioLC (DX-500, Dionex, Sunnyvale, CA, USA; <http://www.dionex.com>) equipped with CarboPac PA-1 column (4 mm \times 25 cm) and a PAD. Size fractionation of α -1,4-glucans was performed with a linear gradient of sodium acetate (50–500 mM) in 0.1 M NaOH at a flow rate of 1 ml/min.

Thermal properties of starch

The gelatinization properties of the purified starch samples were measured using the method of Atichokudomchai et al. (2002) using a Differential Scanning Calorimetry (DSC-650, Scinco, Korea; <http://www.scinco.com>). Briefly, the starch samples were hydrated to a 60% moisture content and each suspension was then transferred to an aluminum pan and hermetically sealed. Following equilibration at room temperature for 1 h, the samples were heated from 25 to 130°C at a heating rate of 5°C/min. An empty aluminum pan was used as a reference and the DSC was calibrated using indium. All measurements were performed three times. The onset (T_o), peak (T_p), conclusion temperature (T_c) and ΔH (crystal melting enthalpy) values of the samples were determined from the DSC thermogram.

Determination of the amylose and amylopectin ratios

The ratio of amylose to amylopectin was determined using the amylose–amylopectin assay kit (K-AMYL,

Megazyme, Ireland; <http://www.megazyme.com>), according to the manufacturer's instructions.

Results

The spatiotemporal expression of the *OsSSIIIa* gene was examined by RT-PCR using gene specific primers. The resulting products revealed that these transcripts are abundant in developing rice seeds, but are barely detectable in other tissues of the rice plant, such as the leaf, root, and flowers (data not shown). RT-PCR analysis was also carried out using cDNAs prepared from a series of rice seeds collected at different development stages (1 to 15 DAF), as well as from ovaries prior to pollination. These experiments revealed that *OsSSIIIa* transcripts are abundant in the caryopsis at 5–15 DAF (data not shown). This finding is consistent with previously reported data (Hirose and Terao 2004; Dian et al. 2005; Ohdan et al. 2005). In addition, we observed that *OsSSIIIa* expression in the leaf is significantly enhanced upon treatment with 175 mM sucrose (data not shown), suggesting that its gene expression is positively regulated in order to increase the starch storage reserve when soluble sugar levels are high. In the present study, we have elucidated a role of *OsSSIIIa* which is distinct from that of *OsSSI* and *OsSSIIa* during starch synthesis in the developing rice endosperm, using two null mutants.

Characterization of two *OsSSIIIa* null mutants

The *OsSSIIIa* gene (TIGR gene locus name LOC_Os08g09230.2; <http://www.tigr.org/tdb/e2k1/osa1/>) of the *japonica* rice variety Nipponbare comprises 16 exons and 15 introns (Fig. 1a). The genomic region of *OsSSIIIa* corresponds to the 15,572–26,742 bp segment of the BAC clone OSJNBa0056O06 (GenBank # AP005441). To further elucidate the role of *OsSSIIIa* in the rice seed endosperm, we isolated two different mutant alleles of this gene. In the first of these alleles, an insertion of pGA2715 T-DNA (Jeong et al. 2002) has occurred in the 11th exon, whereas the second allele harbors a pGA2772 T-DNA insertion (Jeong et al. 2006) in the 1st intron (Fig. 1a). Both mutant alleles are hereafter referred to as *flo5-1* and *flo5-2*, respectively, on the basis of their white-core floury (*flo*) endosperm phenotype, and the causal gene for this phenotype is denoted *OsSSIIIa/Flo5*. The background genotypes of *flo5-1* and *flo5-2*, respectively, are the *japonica* rice cultivars Dongjin and Hwayoung. These *japonica* cultivars are

known not to be significantly different in terms of the morphological phenotypes in their seed endosperm, but do slightly differ in their response to disaster and disease.

The homozygous mutants *flo5-1* and *flo5-2* were isolated by PCR screening from T-DNA segregating progeny populations. In this screen, both gene-specific primer sets (F1/R1 and F2/R2) and T-DNA- and gene-specific primer sets (T1/R1 and T1/R2) were used (Fig. 1b). Of the 10 progeny (T2) individuals of the *flo5-1* line, two appeared to be homozygous for the T-DNA insertion (*flo5-1/flo5-1*), three were wild type (*Flo5-1/Flo5-1*) and five were heterozygotes (*Flo5-1/flo5-1*) (Fig. 1b, left panel). Similar analysis of the *flo5-2* line was performed (Fig. 1b, right panel). We conducted phenotype observations, and found that all homozygous *flo5-1* and *flo5-2* plants exhibited a white-core floury endosperm. Our observations of the heterozygous plants revealed a nearly 3:1 segregation ratio between wild-type and floury endosperms in their progeny, demonstrating that the *flo5* alleles are recessive mutations. For example, among the 138 plants analyzed from the T3 progeny of the *flo5-1* line, 37 floury endosperm seeds were identified as homozygous for the *flo5-1* allele (data not shown). We concluded from these initial data that the floury endosperm phenotype correlates exactly with homozygous *flo5-1* and *flo5-2* genotypes, thus indicating that the mutations in *OsSSIIIa/Flo5* cause the floury endosperm phenotype.

OsSSIIIa/Flo5 expression is barely detectable in different organs of the rice plant, such as the leaf and root, other than the seed endosperm. We therefore performed RT-PCR analysis using cDNAs prepared from endosperm mRNA. This analysis confirmed that *OsSSIIIa/Flo5* transcripts are absent in seed endosperm of the *flo5-1* and *flo5-2* plants, but are present in the wild type endosperm of the Dongjin and Hwayoung parental plants (Fig. 1c). This confirms that the *flo5-1* and *flo5-2* are indeed null mutants of the *OsSSIIIa/Flo5* gene.

Rice grain morphology in the *flo5* mutants

The *flo5* mutants did not exhibit any visibly abnormal phenotypes at the vegetative stage of plant growth and development. As a parallel result, *OsSSIIIa/Flo5* has been found to be expressed preferentially in rice seeds (Hirose and Terao 2004; Dian et al. 2005; Ohdan et al. 2005; data not shown). To determine whether the starch content was altered in these mutants, we measured the starch levels in the flag leaf as well as in the mature rice grains. The results of these analyses did

not show significant differences between the total starch contents of in either the leaves or seeds of the *flo5* mutants (data not shown), indicating that the *OsSSIIIa/Flo5* lesion does not affect transitory starch synthesis in the leaf or total starch content in seed endosperm.

The central portion of the husked rice grains derived from the *flo5-1* and *flo5-2* mutant plants displays a white-core floury endosperm (Fig. 2b, d, left panel), which manifests as a sort of opaque phenotype, whereas the respective wild type control cultivars display a transparent normal endosperm on an illuminator (Fig. 2a, c, left panel). Cross sectioning of the mature mutant rice grains further revealed that the central portion of the endosperm is indeed floury-like, whereas the exterior appears normal (Fig. 2b, d, right panel). This indicates that the disrupted process of starch synthesis resulting from the *OsSSIIIa/Flo5* lesion leads to a deformity of the starch granules. In addition, we examined the effects of the *flo5* mutations on grain weight but did not observe significant differences between the wild type and *flo5* mutant rice (data not shown).

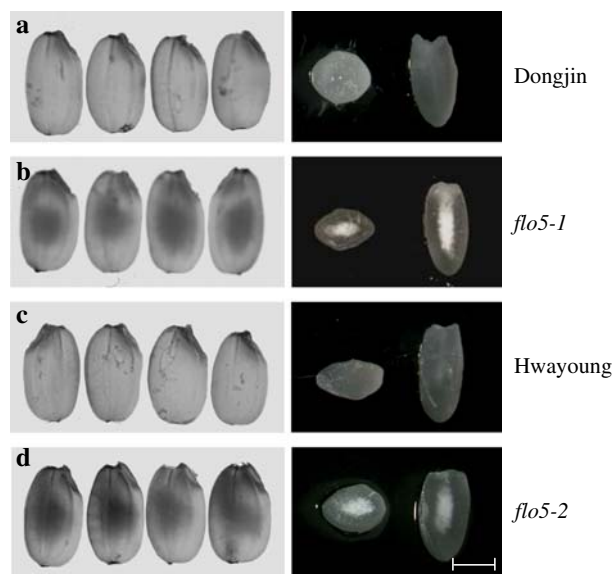


Fig. 2 Morphology of the seed endosperm in the *flo5* mutants (**b, d**) and their respective wild type controls (**a, c**). *Left panels* dehusked seeds of Dongjin **a**, *flo5-1* **b**, Hwayoung **c**, and *flo5-2* **d** placed on an illuminator. Notably, in the *flo5-1* and *flo5-2* seed endosperms, a dark central portion is commonly evident and is characteristic of a white-core floury endosperm. *Right panels* horizontal and longitudinal sections of polished seed endosperms of *flo5* mutants (**b, d**) and wild type rice plants (**a, c**). A central white-core floury endosperm is prominent in the *flo5* mutants. *Bar* 2 mm

Morphology of the starch granules in the *flo5* mutants

To examine starch granule morphology, we performed SEM analysis using cut seed endosperm and purified starch granules (Fig. 3). In these experiments, cross-sectioned endosperm of polished rice grains revealed that the *flo5* mutants have a loosely packed central portion (Fig. 3b, d, top and middle panels), probably due to their abnormal starch granule morphology. In contrast, the wild type control endosperm was found to be occupied by densely packed starch granules in the central portion (Fig. 3a, c, top and middle panels). The starch of both Dongjin and Hwayoung wild type cultivars was found to form as polygonal granules with a sharp edge, characteristic of the typical starch granule morphology in normal rice endosperm (Fig. 3a, c, bottom panel). In contrast, in the *flo5-1* and *flo5-2* mutants, the starch granules were observed to be smaller than that of the parental controls, and did not display the sharp edge seen in the wild type starch granules (Fig. 3b, d, bottom panel).

X-ray diffraction analysis of starch granules

Starch is a semi-crystalline biopolymer containing both amorphous and crystalline structures with a different crystal type. It has been shown that the endosperm starch in rice grains exhibits the A type crystal pattern (Buléon et al. 1998). We performed X-ray

diffraction analysis using endosperm starch from both the wild type controls and *flo5* mutants and confirmed that they all display a typical A type crystal pattern with four peaks at 15.06, 17.16, and 17.94, and 23.00 of diffraction angle 2θ (Fig. 4). The starch granules in the *flo5-1* and *flo5-2* mutants, however, showed a relatively lower peak intensity than their respective wild type counterparts, Dongjin and Hwayoung (Fig. 4; Table 1). Hence, our X-ray diffraction analysis is consistent with our earlier findings that the loss of the *OsSSIIIa/Flo5* gene function causes an impaired starch crystalline structure, resulting in a relatively lower crystallinity in *flo5* mutants but no change in the crystal type.

Chain length distribution analysis of endosperm starch in *flo5* mutants

To further understand the role of *OsSSIIIa/Flo5* during the formation of starch endosperm, the chain-length distribution of isoamylolysates of endosperm amylopectin was determined by HPAEC-PAD in both the *flo5* mutants and control lines (Fig. 5). In the *flo5-1* and *flo5-2* mutant lines, starch chains with \geq DP 30 were found to be commonly decreased. This strongly indicates that *OsSSIIIa/Flo5* plays an important role in the generation of relatively long chains (B_2 and B_3 to B_4). In addition, chains with DP 9 to 15 and DP 22 to 29 were increased in *flo5-1* and *flo5-2*, whereas chains with DP 6 to 8 and DP 16 to 20 were

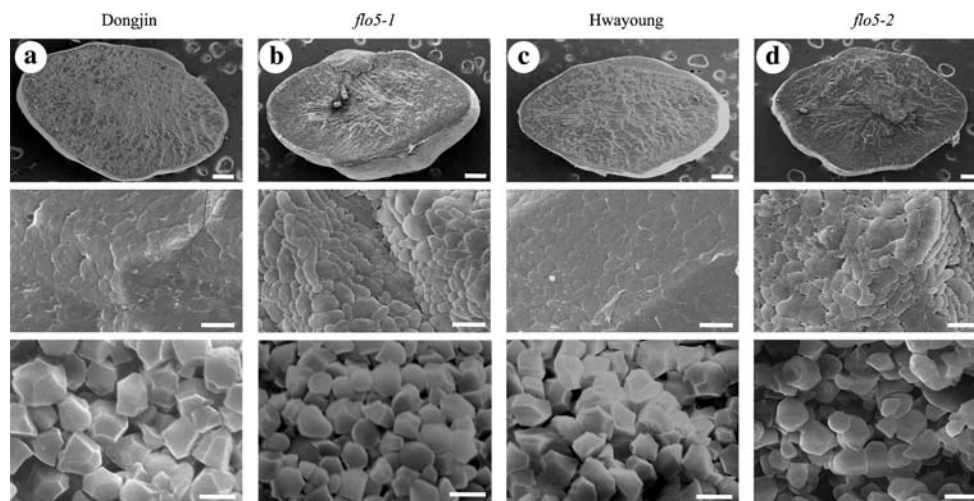


Fig. 3 Scanning electron micrographs of cut endosperms and endosperm starch granules of *flo5-1* and *flo5-2* (b, d) and their respective wild type controls, Dongjin and Hwayoung (a, c). *Top and middle panels*, cutting planes of seed endosperms. The *flo5* mutant endosperms appear to be loosely packed in the central

portion when compared to their wild type controls. *Bottom panels* starch granules in the *flo5* mutants are smaller and rounder with less sharpness, compared with their respective wild type controls. *Bar* 300 (top), 10 (middle) or 5 μ m (bottom)

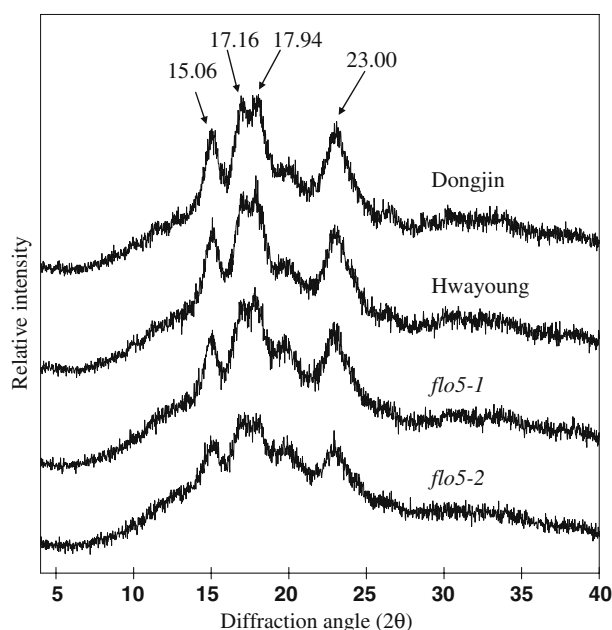


Fig. 4 X-ray diffraction patterns of endosperm starch granules isolated from the *flo5-1* and *flo5-2* mutants and their wild type Dongjin and Hwayoung controls. The relative crystallinity of the *flo5-1* and *flo5-2* starch is decreased when compared with the controls

Table 1 Comparison of the relative crystallinity of the endosperm starch between the *flo5-1* and *flo5-2* mutants and their respective wild type controls

Genotype	Relative crystallinity (%) ^a	Relative crystallinity ratio (%)
Dongjin	10.14	100
Hwayoung	9.34	100
<i>flo5-1</i>	8.14	80.3
<i>flo5-2</i>	5.63	60.3

*I*_a Amorphous area on the X-ray diffractogram, *I*_c crystallized area on the diffractogram

^a Relative crystallinity (%) = $I_c / (I_a + I_c) \times 100$

decreased in these mutants. It is thus likely that the changes in the relatively short chains of amylopectin in the *flo5* mutants can be attributed to altered activities of other SS isoforms. We further examined whether similar changes in chain length distribution patterns occurred in other white-core floury endosperm mutants. We analyzed *flo4-1* and *flo4-2* mutants in this regard, since *Flo4* was shown previously to encode a C-type pyruvate orthophosphate dikinase (OsPPDKB), rather than a starch synthesis-related enzyme (Kang et al. 2005). We found no significant differences in the chain length distribution pattern in the *flo4* mutants and their respective control lines (data not shown).

Thermal properties of endosperm starch in the *flo5* mutants

To evaluate the thermal properties of starch harboring an altered structure and composition, the gelatinization temperatures of the endosperm starch in the *flo5* mutants and wild type plants were analyzed by DSC. The results of this analysis show that the gelatinization temperatures at the onset (T_o), peak (T_p), and conclusion (T_c) in *flo5* endosperm starch differ from wild type (Table 2), and are approximately 1–5°C lower compared with the control lines. This indicates that modified starch structure and reduced crystalline region in *flo5* mutants decrease the gelatinization temperature of endosperm starch. The *flo5* mutants also exhibit slightly higher ΔT than the controls, which is consistent with our finding that the proportion of the long chains in amylopectin is reduced in these mutants when compared with wild type. It is known that the reduced content of long chains of amylopectin lowers T_o gelatinization temperature, thereby increasing ΔT (Patindol and Wang 2003).

In addition, the ratio of amylose to amylopectin appeared to be approximately 2–4% increased in the starch samples isolated from *flo5* mutants when compared with their controls (Table 3). The minor increase in amylose content of the mutant endosperm is possibly due to either reduced synthesis of amylopectin or increased synthesis of amylose.

Discussion

In the present study, we find that OsSSIIIa/Flo5 plays a critical role during starch synthesis in the seed endosperm of rice and that this is distinct from other SS enzymes. Analysis of null mutants of this gene demonstrates that the loss of OsSSIIIa/Flo5 activity is not fully compensated by the functions of other SS enzyme activities. The *flo5* mutants manifest a visible white-core floury endosperm phenotype, due to insufficient crystallized amylopectin formation and a modified starch granule.

Characterization of *OsSSIIIa/Flo5* mutants

The importance of SSIII activity during amylopectin biosynthesis is supported by the results of previous investigations of maize endosperm (Gao et al. 1998; Jane et al. 1999), potato tuber (Edwards et al. 1999; Lloyd et al. 1999), and the *Arabidopsis* leaf (Zhang et al. 2005). An antisense construct was successfully introduced to reduce the activity of the major isoform

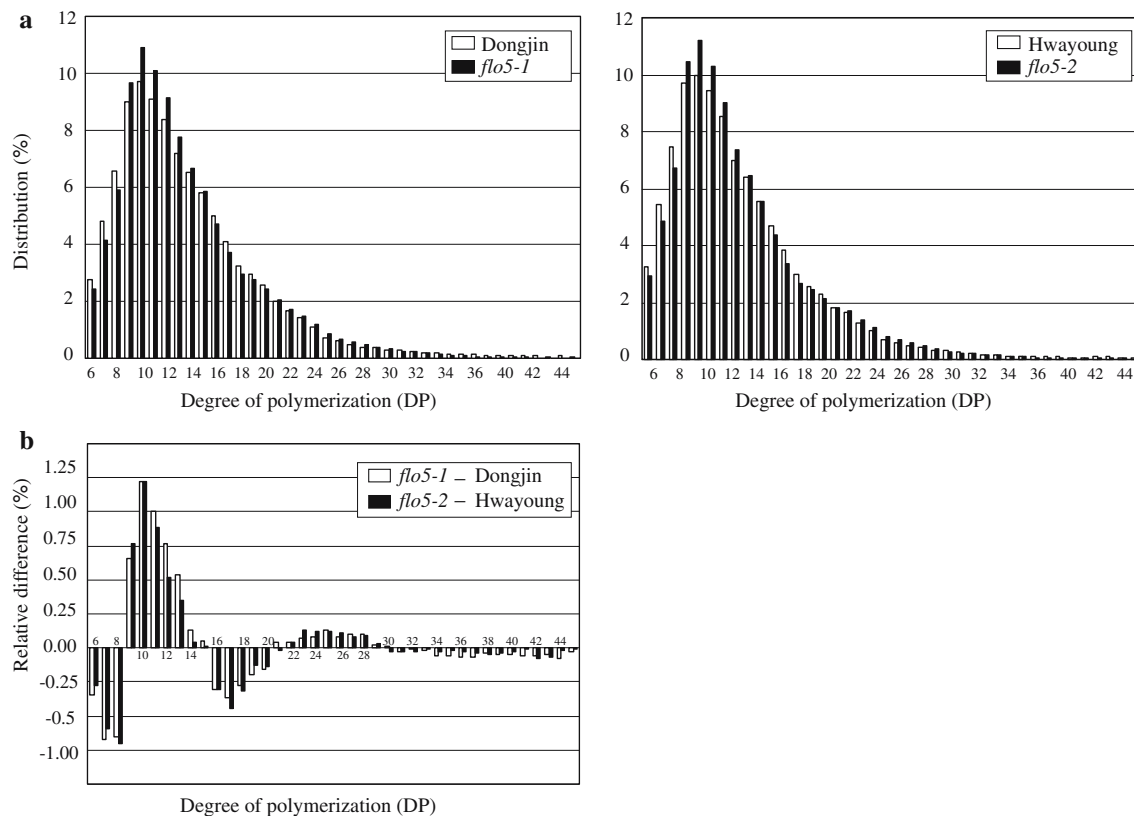


Fig. 5 Chain-length distribution of amylopectin isolated from the endosperm of *flo5* mutants and wild type controls. **a** Chain length distribution of debranched amylopectin from rice endosperm of *flo5-1* (filled square) and its wild type control Dongjin (open square) (left panel), and of *flo5-2* (filled square) and its wild type control Hwayoung (open square) (right panel). The number under the bars indicates the degree of polymerization (DP). Note that there are no significant differences in the distribution of chains between two wild type cultivars or between the two mutant alleles. Areas of each peak are expressed as the

percentage of the sum of peak areas from DP 6 to DP 45. **b** Relative differences in the chain lengths of amylopectin between *flo5-1* and its wild type counterpart Dongjin (open square), and between *flo5-2* and its wild type control Hwayoung (filled square). Relative differences are expressed as the percentage of peak areas of *flo5-1* and *flo5-2* subtracted from those of Dongjin and Hwayoung, respectively. Chains with DP 6 to 8, DP 16 to 20, and \geq DP 30 are shown to be decreased, whereas DP 9 to 15 and DP 22 to 29 appear to be increased in the *flo5* mutants

Table 2 Thermal properties of endosperm starch from *flo5-1* and *flo5-2* mutants and the respective wild type controls

Genotype	T_o	T_p	T_c	ΔT	ΔH
Dongjin	$57.3 \pm 0.3^*$	65.1 ± 0.3	80.7 ± 1.7	23.5 ± 1.5	5.5 ± 0.8
Hwayoung	55.5 ± 1.6	64.2 ± 0.1	81.5 ± 1.3	26.0 ± 2.6	6.1 ± 2.2
<i>flo5-1</i>	53.6 ± 0.3	60.0 ± 1.1	81.3 ± 0.6	27.8 ± 1.3	5.9 ± 1.8
<i>flo5-2</i>	54.1 ± 0.6	61.1 ± 0.4	82.1 ± 1.6	28.0 ± 1.9	5.6 ± 0.4

* Mean \pm SD

T_o , onset temperature; T_p , peak temperature; T_c , conclusion temperature; ΔT , $T_c - T_o$; ΔH , crystal melting enthalpy

Table 3 Amylose contents in endosperm starch from *flo5-1* and *flo5-2* mutants and the respective wild type controls

Genotype	Amylose (%)
Dongjin	$19.29 \pm 0.29^*$
Hwayoung	19.26 ± 0.82
<i>flo5-1</i>	21.96 ± 0.69
<i>flo5-2</i>	23.46 ± 0.52

* Mean \pm SD

of SSIII in potato tubers (Edwards et al. 1999; Lloyd et al. 1999). In tubers of the *SSIII* antisense transgenic line, the chain length distribution pattern revealed a decrease in relatively long chains of approximately DP 20 to 35, resulting in the increased production of short chains. Thus, it was suggested that SSIII functions in the synthesis of relatively long chains. An increase in the rate of transitory starch accumulation during the

light phase of a diurnal cycle was observed in the *Arabidopsis SSIII* mutants, *Atss3-1* and *Atss3-2* (Zhang et al. 2005). The increased starch synthesis in these mutants was assumed to be due to the removal of an inhibitory effect of SSIII upon the activities of other starch biosynthetic enzymes. From the analysis of the chain length distribution in *Arabidopsis* leaf starch, the *Atss3-1* and *Atss3-2* mutants were found to contain higher quantities of amylopectin of \leq DP 10, which represents unbranched external A chains, and reduced levels of approximately DP 11 to 22. In particular, very long chains estimated to be \geq DP 60 were more prevalent in the mutant starch.

Of the eight soluble *SS* genes identified, *OsSSIIIa/Flo5* belongs to the late expresser group, suggesting that it plays a more predominant role at the starch filling phases than in the early pre-storage phase (Hirose and Terao 2004; Dian et al. 2005; Ohdan et al. 2005; data not shown). This expression pattern is consistent with the fact that the phenotypes associated with the *flo5* alleles do not differ from their wild type counterparts in terms of starch content in the leaf and also during plant growth prior to seed setting. Likewise, the white-core floury endosperm phenotype in the *flo5* mutants indicates that the abundant expression of the *OsSSIIIa/Flo5* gene at the starch filling phase correlates well with the requirement for the OsSSIIIa enzyme during normal starch synthesis. Our chain length distribution pattern analysis further indicates that the mutations in this gene cause a reduction in the relatively long chains of \geq DP 30, indicating that rice SSIIIa largely functions in the elongation of long chains of amylopectin, as also seen in antisense SSIII potato transgenic plants (Edwards et al. 1999; Lloyd et al. 1999) and the maize *dull-1* mutant (Gao et al. 1998). Our chain length distribution analysis also found additional changes in the amylopectin structure of the *flo5* mutants, including a decrease in chains with DP 6 to 8 and DP 16 to 20 and increase in chains with DP 9 to 15 and DP 22 to 29. This is not particularly surprising, given the fact that null mutants for specific starch synthesis gene may manifest alterations in the activities of other SS enzymes, thereby causing somewhat complex changes in their chain distribution pattern of amylopectin (see below).

A distinct function of OsSSIIIa/Flo5 during starch synthesis in rice endosperm

OsSSI and OsSSIIIa contribute to a large portion of the overall SS enzyme activity in the developing endosperm in rice (Fujita et al. 2006). A similar

observation was observed in the soluble fraction of developing maize endosperm (Cao et al. 1999), indicating the importance of the function of these isoforms in the synthesis of endosperm starch. Measurements of dissociation constants further indicated that during amylopectin synthesis, shorter A chains (DP 6 to 12), which carry no other chains and are linked via their reducing ends, and B₁ chains (DP 13 to 24; Hanashiro et al. 1996), are extended by OsSSI up to a DP 20 chain length. This chain thus becomes unsuitable for catalysis by OsSSI and therefore cannot be elongated further by this enzyme (Commuri and Keeling 2001). This suggests that SSI become entrapped as a relatively inactive protein within the starch granule. Using Tos17-tagged mutants in rice, OsSSI appears to generate DP 8 to 12 chains from short DP 6 to 7 chains emerging from the branch point in amylopectin chains A or B₁, suggesting that SSI has a distinct role in elongating very short chains (Fujita et al. 2006). Thus, further glucan extension for the continuation of amylopectin synthesis requires the activity of other SS enzymes that can generate longer glucan chains.

OsSSIIa is one of the SS enzymes whose gene expression is abundant during the starch filling phase (Hirose and Terao 2004; Ohdan et al. 2005; data not shown). It has also been shown that OsSSIIa from an *indica*-type rice is an active isoform, whereas the *japonica*-type of this isoform has less activity in synthesizing amylopectin (Umamoto et al. 2002; Jiang et al. 2004; Nakamura et al. 2005). These differences in the activity of OsSSIIa between *indica*- and *japonica*-type cultivars have now been shown to be attributable to four variable amino acids (Nakamura et al. 2005). In the same previous study, the *indica*-type OsSSIIa was also found to elongate chains with DP 6 to 11 to chains with DP 13 to 28. Similar results have been observed in the maize *sugary2* (Zhang et al. 2004), barley *sex6* (Morell et al. 2003), wheat *GSP-1 null* (Yamamori et al. 2000), and antisense *SSII* potato plants (Edwards et al. 1999; Lloyd et al. 1999).

In the present study, on the basis of our mutant analysis, we speculate that OsSSIIIa contributes in vivo to the amylopectin synthesis of chains with \geq DP 30 from intermediate chains, preferentially with DP 16 to 20, in rice endosperm. This finding is consistent with the previous in vitro results from chain-length analysis of amylopectin in the OsSSIIIa band excised from a native polyacrylamide gel electrophoresis/SS activity staining gel (Fujita et al. 2006). In our in vivo assays, it appears that OsSSIIIa synthesizes amylopectin with a broad range of long chains and that the decreased short chains of DP 6 to 8 in the *flo5* mutant alleles might be due to increased SSI activity. This would contribute in

part to the increased synthesis of chains with DP 9 to 15 from very short chains with DP 6 to 8. Simultaneously, the loss of OsSSIIIa/Flo5 function might enhance endogenous OsSSIIa activity, thereby facilitating the synthesis of both DP 9 to 15 and DP 22 to 29. As supporting evidence, SSI activity in the maize SSIII mutant *dull-1* was shown previously to be higher than that of the wild type (Cao et al. 1999). In *Arabidopsis* SSIII mutants, total SS activity was also found to be increased, possibly owing to a modification of the SS enzymes (Zhang et al. 2005). Similarly, SSIIIa activity in *OsSSI* mutants appears to increase compared with the control lines (Fujita et al. 2006). Therefore, an interaction among the OsSS enzymes is likely to partly compensate for a deficiency of a specific SS isoform. Detailed analysis of other SS isoforms will be needed to properly elucidate this possibility. Furthermore, SS activity may not be the only factor contributing to chain length distribution, as BE or DBE activity can also alter the characteristics of starch polymers.

Together with previous results, the findings of our current study provide direct evidence that amylopectin chains in the rice endosperm are synthesized principally by the coordinated actions of three enzyme isoforms, OsSSI, OsSSIIa, and OsSSIIIa/Flo5. We surmise also that during starch synthesis in rice endosperm, OsSSI contributes to the initial short chain elongation of DP 6 to 7, of the A or B₁ chain produced by BE, to DP 8 to 12 chains, and that its activity persists from the beginning to the maturation stage of endosperm development. OsSSIIa, which is abundant at the starch storage phase after 5 DAF, is then involved in the elongation of DP 6 to 11 to chains with DP 13 to 28. Subsequently, OsSSIIIa/Flo5 activity, which is also abundant at the starch filling stage, is a prerequisite for further elongation of the intermediate chains to \geq DP 30 chains. The functions of the remaining fourth group of SSIV isoforms remain to be characterized.

Malformed starch granules in *flo5* mutants result in a white-core floury endosperm

The *flo5* mutants display loosely packed starch granules that eventually lead to a white-core floury endosperm. Similarly, the maize *SSIII* mutant displays this dull phenotype (Gao et al. 1998). In contrast, in *OsSSI* mutants, changes in the chain distribution patterns are observed but the starch granules maintained their normal structures (Fujita et al. 2006). Moreover, in *OsSSI* mutants, short chains were found to be commonly reduced in their distribution pattern but

relatively long chains are somewhat increased (Fujita et al. 2006), which differs from our current observations of the *flo5* mutants which show reduced long chains of \geq DP 30. This suggests that the reduction of short chains in amylopectin may not significantly affect the starch granule morphology, since relatively long chains are increased by altered starch synthesis activities in *OsSSI* mutants.

We demonstrate in the present study that the constituent chain lengths within amylopectin, and the structure and properties of starch granules are altered when the function of OsSSIIIa/Flo5 is removed in rice endosperm. This study also shows that a using single knockout line can be a very effective approach to the unveiling of the distinct roles of SS isoforms in determining the complex structure of amylopectin. To further understand the coordinated actions of these starch synthesis-related enzymes in rice endosperm, more detailed analysis of the interactions between these isoforms will be needed.

Acknowledgments This work was supported, in part, by grants from the SRC for the Plant Metabolism Research Center (PMRC), Korea Science and Engineering Foundation (KOSEF) Program; from the Biogreen 21 Program, Rural Development Administration; from the Crop Functional Genomic Center (CG1422), the 21 Century Frontier Program; and from the BK21 program, Ministry of Education and Human Resources Development.

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