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ORIGINAL ARTICLE

The impact of the *indica* rice *SSIIa* allele on the apparent high amylose starch from rice grain with downregulated japonica *SBEIIb*

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Abstract

Key message **Catalytically active indica SSIIa allele in high amylose rice with down-regulated japonica SBEIIb can increase starch content and modify the starch structure and properties without changing its amylose content.**

Abstract Rice (*Oryza sativa*) genotypes with inactive starch synthase IIa (SSIIa) with recessive variants of starch branching enzyme IIb (SBEIIb) exhibit a range of alterations in grain phenotype, starch granule morphology, starch granule bound proteins, starch structure, and functional properties. However, the interactions between the two enzymes have not been thoroughly investigated yet. We analysed recombinant rice lines having down-regulated *SBEIIb* expression ($SBEIIb^{DR}$) with either *indica* or *japonica* type *SSIIa* ($SSIIa^{ind}$ or $SSIIa^{jap}$). In $SBEIIb^{DR}$ rice starch granules, the increased abundance of two protein bands (SSI and SSIIa) was found with eight additional protein bands not generally associated with starch granules. The amount of SSIIa was higher in $SSIIa^{ind}SBEIIb^{DR}$ than $SSIIa^{jap}SBEIIb^{DR}$, which indicated that *indica* type SSIIa, possibly in the monomer form, was extensively involved in starch biosynthesis in the $SBEIIb^{DR}$ endosperm. Furthermore, $SSIIa^{ind}SBEIIb^{DR}$ grains had higher total starch content and higher starch swelling power than $SSIIa^{jap}SBEIIb^{DR}$ *lines*, but the amylopectin gelatinization temperatures and enthalpy and the apparent amylose content remained similar. In summary, this work suggests that $SSIIa^{ind}$ can partly compensate for the alteration of starch synthesis resulting from the *SBEIIb* down-regulation in *japonica* background without reducing its amylose content. The study provides insight into the starch structural and textural improvements of high amylose starch.

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Background

Amylose and amylopectin are the two principal components of starch. The former molecule (20–30% dry weight of starch) is a long linear glucose polymer with few branches. It is mainly elongated by adding glucose from ADP-glucose to existing α-(1,4)-glucan chains catalysed by granule bound starch synthase I (GBSSI). GBSSI is encoded by *Wx,* which primarily determines the amylose content of starch in cereals (Nelson and Rines 1962; Tsai 1974; Fedoroff et al. 1983; Shure et al. 1983). Amylopectin typically makes up 70–80% of rice (*Oryza sativa*) starch and is a much larger molecule with frequent α-(1–6)-branches. Amylopectin is synthesized by multiple enzymes in a coordinated manner, involving starch synthases (SSs), starch branching enzymes (SBEs), debranching enzymes (DBEs), starch phosphorylase and other regulatory proteins (Ball and Morell 2003; Martin and Smith 1995). Distinctive SSs isoforms (SSI, SSII, SSIII, and SSIV) act in succession to elongate the amylopectin linear glucan chains. Isoforms of SBEs (SBEI and SBEII)

play distinct roles in the formation of branch linkages in amylopectin (Ball and Morell 2003). DBEs catalyse the debranching of α-(1–6)-glucan chains of amylopectin and removal of improperly linked branches.

Among these enzymes, active SSIIa and SBEIIb are both crucial for maintaining the regular chain length of amylopectin in rice (*Oryza sativa*) grains. SSIIa is responsible for the elongation of short α-(1,4)-glucan chains (DP < 10) of amylopectin to reach intermediate chain length (11 < DP < 24) (Umemoto et al. 2002, 2004). On the other hand, SBEIIb transfers short chains (DP 6–7) to form α-(1,6)-glucosidic linkages on the outer chains of amylopectin (Nakamura et al. 2010). Previous studies indicated that *japonica* type SSIIa ($SSIIa^{jap}$, a less-active isoform), in contrast to *indica* SSIIa ($SSIIa^{ind}$, an active isoform), led to amylopectin with increased short chains and decreased intermediate chains (Umemoto et al. 2002, 2004). Although the variation in amylopectin structure, the apparent amylose content (AAC) of these starches is comparable (Umemoto et al. 2002, 2004). Neither of the two SSIIa isoforms causes significant differences in starch granule or grain morphology in the hybrid rice (Luo et al. 2015a) or near-isogenic lines (Umemoto et al. 2008). On the contrary, *SBEIIb* recessive rice variants (*amylose-extender* mutants (*ae*) and *SBEIIb* down-regulated transgenic lines) produce fewer short chains and more intermediate chains of amylopectin than wildtypes in rice grains both in *indica* and *japonica* backgrounds (Nishi et al. 2001; Butardo et al. 2011, 2012). The increase of starch AAC of rice SBEIIb mutant is due to the consequence of increased long-chain relative proportion in amylopectin molecules, rather than amylose increase (Butardo et al. 2011). The starch granules of rice *SBEIIb*-recessive and down-regulated grains are round-shaped and loosely present in their compound structure, and the grains are smaller and chalky or opaque (Nishi et al. 2001; Butardo et al. 2011; Sun et al. 2017).

SSIIa and SBEIIb proteins are believed to be involved in amylopectin biosynthesis in a trimeric protein complex together with SSI protein in wheat (Tetlow et al. 2008) and maize (Hennen-Bierwagen et al. 2008). Analysis of the maize *sugary-2* mutant demonstrated that SSIIa protein is closely associated with SBEIIb and SSI proteins and links with starch granules during starch synthesis in the endosperm. Therefore, it is believed that SSIIa protein plays the role of the core in the formation of the trimeric protein complex of SSI-SSIIa-SBEIIb in maize (Liu et al. 2012). The *sugary-2* starch granules are found to be devoid of amylopectin biosynthetic enzymes (Liu et al. 2012). That study strongly supports the previous reports on significantly decreased amounts of SBEIIb and SSI inside cereal starch granules in the endosperm resulting from either reducing the activity or eliminating the presence of SSIIa (Yamamori et al. 2000; Morell et al. 2003; Umemoto et al. 2004; Umemoto and Aoki 2005; Kosar-Hashemi et al. 2007; Grimaud et al. 2008). It appears that SSIIa deficiency results in an altered granular distribution pattern of SSI and SBEIIb, with both proteins remaining in the amyloplast stroma instead of associating with starch granules after synthesis (Luo et al. 2015b).

The interactions among SSI, SSIIa and SBEIIb in enzyme complexes of cereal endosperm have been widely investigated using co-immunoprecipitation methods in barley (Ahmed et al. 2015), wheat (Tetlow et al. 2004, 2008), maize (Hennen-Bierwagen et al. 2008, 2009; Liu et al. 2009, 2011, 2012), and rice (Crofts et al. 2015, 2018). Interactions among SSI, SSIIa, and SBEIIb in rice have been mainly investigated by analysing multiple-gene mutants. Abe et al. (2014) reported that an *SSI* and *SBEIIb* double-null mutant was rarely fertile, but a double mutant with *SSI*-leaky mutation and *SBEIIb*-null had opaque seeds. The branching activity of SBEIIb in amylopectin chain formation is followed by the SSI activity of chain elongation (Nakamura 2002). An in vitro study of SSs and SBEs indicated that SBEIIb stimulates SSI activity in glucan chain synthesis by increasing the number of non-reducing ends and functional interaction with SSI in rice endosperm (Nakamura et al. 2014). It was also postulated in a study analysing starch structure that SS (SSI or SSIIIa) deficiency alters the branch points of side chains via the effects on enzyme complex formed by SS and SBE (Hanashiro et al. 2011). It has been demonstrated that ten starch synthetic enzymes are present at larger molecular weights of protein complexes than their respective monomeric sizes through various interactions of different enzymes, including SSI, SSIIa, and SBEIIb (Crofts et al. 2015, 2018). However, the functional interaction between SSIIa and SBEIIb and corresponding starch properties have not been reported in a double gene mutant so far.

In this study, we investigated the impacts of $SSIIa^{ind}$ and $SSIIa^{jap}$ alleles on *SBEIIb* down-regulated allele ($SBEIIb^{DR}$) in rice grain on starch structures and properties. Rice grain with $SSIIa^{ind}$ and $SBEIIb^{DR}$ alleles produced starch with novel starch structure and properties. The role of *SSIIa* alleles is discussed with regards to rice starch quality and functional property improvement.

Materials and methods

Plant materials

All plants were grown in the glasshouse of plant growth facility of CSIRO Agriculture and Food (Canberra) at 27 °C under natural light conditions. The experimental rice lines were derived from a cross-population between two parental rice lines (A203 and 3–12). The line A203 was a rice line with down-regulated *SBEIIb*

gene expression ($SBEIIb^{DR}$) in a Nipponbare background through the artificial microRNA technology (Butardo et al. 2011). The line 3–12 was an inbred line having heterozygous SSIIa allele ($SSIIa^{ind}SSIIa^{jap}$) and homozygous $SBEIIb^{jap}$allele ($SBEIIb^{jap}SBEIIb^{jap}$), which was derived from a crossing population between a *japonica* variety (Nipponbare) and an *indica* variety (IR64) (Luo et al. 2015a). Both A203 and 3–12 lines contained *japonica* type *Wx* locus to ensure the same effect of *Wx* locus on amylose content in all progeny lines.

The derivation of recombinant lines is shown in Fig. 1. The F2 seeds were separated into two groups according to the phenotype. The opaque grains were verified to be homozygous $SBEIIb^{DR}$ allele ($SBEIIb^{DR}SBEIIb^{DR}$) or heterozygous $SBEIIb^{DR}$ allele ($SBEIIb^{WT}SBEIIb^{DR}$). In contrast, the translucent grains were homozygous wild type *SBEIIb* allele ($SBEIIb^{WT}SBEIIb^{WT}$) using gene-specific markers (Butardo et al. 2011). The DNA markers used to determine the genotypes of *SSIIa* were published in our previous work (Luo et al. 2015a). After 3–4 generations of phenotyping and genotyping selections, the homozygote lines were segregated into four groups (G1 to G4) by two alleles of *SSIIa* and *SBEIIb* genes: G1, $SSIIa^{ind}SSIIa^{ind}/SBEIIb^{DR}SBEIIb^{DR}$; G2, $SSIIa^{jap}SSIIa^{jap}/SBEIIb^{DR}SBEIIb^{DR}$; G3, $SSIIa^{ind}SSIIa^{ind}/SBEIIb^{WT}SBEIIb^{WT}$; G4, $SSIIa^{jap}SSIIa^{jap}/SBEIIb^{WT}SBEIIb^{WT}$. Grains of three independent F4 lines were used as triplicates for each group in the following analysis.

Grain weight

Grain weight for one line of each four groups was determined as the average grain weight of 20 grains with three replicates.

Microscopic examination of rice grains

The dehulled rice grains from one line of each four groups were photographed with a Leica MZFLIII (Leica Microsystems, Germany) at 12.5 amplification.

Starch preparation

Mature panicles were harvested and dried at 37 °C. The seeds were threshed manually and dehulled with a Satake dehuller. Brown rice grains were then polished using a fabricated circulating abrasive polishing machine. Polished grains (approximately 100–150 mg) were ground in a capsule with ball bearing of an ESPE CapMix™ (model 3 M, AU) for 30 s three times to produce flour samples. The flour samples were then washed with 0.005% NaOH by vigorous vortexing for 2 min, followed by filtration through 0.5 mm nylon sieves. Each sample was then washed three times by vortex mixing and centrifugation at 5000 g for 5 min. The starch pellet was resuspended in a phosphate buffer (50 mM, pH7.5) containing proteinase K (50 µg/ml) and incubated at 37 °C for 2 h. After 5 min centrifugation at 5000 g, the pellet was suspended in water and centrifuged, and the water washing process was repeated three times. The starch pellet was then washed with acetone once and centrifuged for 5 min centrifugation at 5000 g. The residual starch was air-dried at 37 °C overnight.

Starch granule bound protein analyses

Starch samples were used for granule bound proteins (GBPs) extraction, as previously described (Luo et al. 2015a). The total crude proteins were then separated by SDS-PAGE, and the gels were stained for visualization. Afterwards, the protein bands were excised for mass spectrometry (MS) analysis

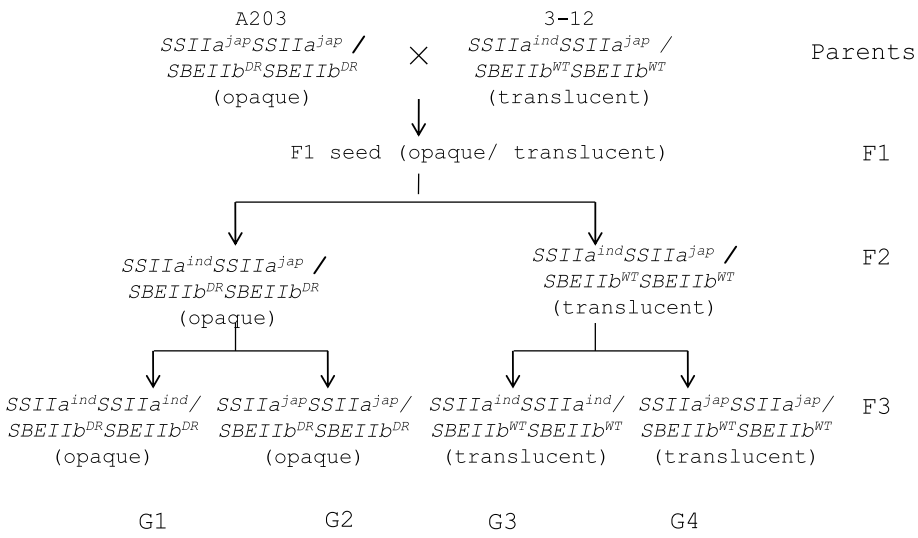

Fig. 1 Generation of four genotype groups of rice lines for SBEIIb and SSIIa alleles. The parents are ami-SBEIIb (A203) and L3-12. The derivation of self-pollinated progenies is indicated by lines with arrowheads. The genotype of *SSIIa* and *SBEIIb* in each line is indicated, and the grain phenotype in brackets. Generations (from Parents to F3) are labeled on the right, and four groups are labeled underneath. *Ind*: indica; *Jap*: japonica; DR: down-regulated; WT: wildtype

(Luo et al. 2015a). The gels were also immunoblotted using specific antisera, as described in our previous work (Luo et al. 2015a).

Starch structural analyses

Total starch content (TSC) of rice flour samples was determined using a Megazyme total starch analysis kit. The analyses were adapted to a 96-well microplate following the manufacturer's procedure (AACC Method 76.13). Rice flour samples were also used for the following analyses: apparent amylose content (AAC) by iodometric estimation (Konik-Rose et al. 2007), chain length distribution (CLD) of debranched starch by fluorophore-assisted carbohydrate electrophoresis (FACE) (O'Shea et al. 1998) and size-exclusion chromatography (SEC). The procedures were conducted following published methods (Luo et al. 2015a).

Starch property analyses

Flour samples were used to measure the swelling power of starch following the method of Konik-Rose et al. (2001). A Differential Scanning Calorimeter (DSC 8000, PerkinElmer, USA) was used to determine the thermal profiles of triplicate flour samples for each line. The procedures were conducted following published methods (Luo et al. 2015a). Data were analysed using the instrument software provided by the manufacturer.

Microscopic and particle size analysis of separated starch granules

Starch samples of each group were spread on the surface of a carbon stub and coated with gold. The morphology of the starch granules was observed under an environmental scanning electron microscope (SEM) (Zeiss EVO LS15) at room temperature and 10 Pa pressure.

Granule size distribution (by volume) of the starch slurries was determined using a laser-diffraction particle size analyser (Mastersizer 2000, Malvern Instruments, Malvern, England). The percentage of starch granules was calculated using a cut-off diameter of starch granules at < 1.9 µm, < 10 µm, and < 20 µm.

Statistical analyses

Statistical analyses were performed using one-way Analysis of Variance of Genstat version 9. Analysis of variance was performed to obtain the least significant differences at $p < 0.05$, looking at variations among groups.

Results

Starch granule bound protein analyses

SDS-PAGE analysis showed that two parental lines (Nipponbare and A203) had different protein banding patterns. Using crude protein extract from Nipponbare starch, four protein bands were separated by SDS-PAGE at 60 kDa and above (Fig. 2). These protein bands were identified by MS analysis as SSIIa, SBEIIb, SSI, and GBSSI, from high to low molecular weight, respectively. This result was corroborated by our previous observation (Luo et al. 2015a). In contrast, other new granule bound proteins were detected in the *SBEI-Ib*^{DR} line (A203). The MS analysis identified phosphatidylinositol 4-kinase (PI 4-kinase), pullulanase, SBEI, and 70 kDa heat shock (Hsp70) proteins (Fig. 2; Supplemental Fig. 1). Three protein bands could not be identified because of their low abundance and/or absence in the Uniprot database (band 1, 4, and 9 indicated by question marks, Fig. 2).

Immunoblot and SDS-PAGE analyses showed that four genotype groups of grains contained different protein banding patterns in starch granules. Immunoblot

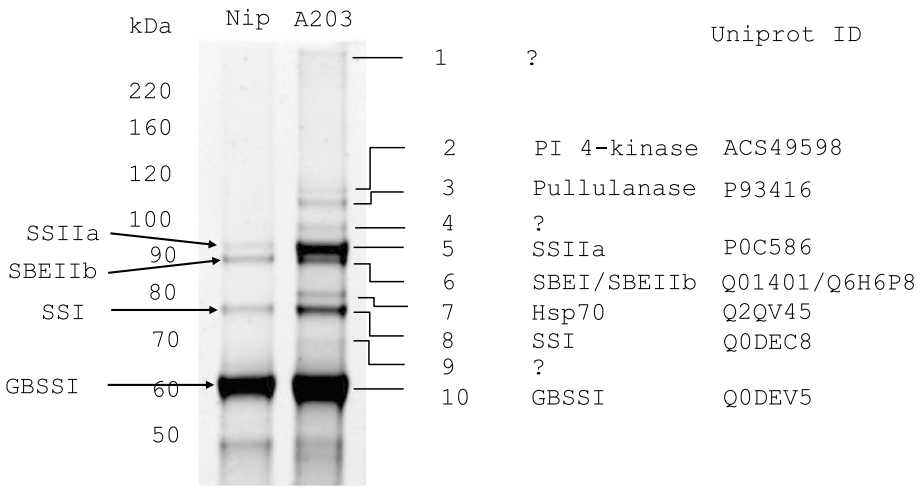

Fig. 2 Analysis of starch granule bond proteins in mature rice grain starch of ami-SBEIIb (A203) and Nipponbare (Nip) by SDS-PAGE. The molecular sizes are labeled on the left of the gel in kDa. The bands are numbered as 1–10 based on their positions from top to bottom of the gel. The identity of each protein bands in each sample is indicated by lines on the right side, followed by Uniprot ID

analysis using specific antisera showed that G1 genotype group had a high abundance of SSIIa protein. In contrast, the SSIIa protein abundance was low in G2 and G3, and absent in G4 (Fig. 3a). Similarly, SBEI and SBEIIa proteins were observed in both G1 and G2 samples using specific antibodies, and their protein abundance was just slightly higher in G1 than G2. The SBEI protein bands in G2 were very low abundance, which was hard to be observed. SBEI protein could not be detected in both G3 and G4, while SBEIIa protein was present at a low level. In contrast, SBEIIb protein was observed in G3 and G4, and G3 had higher SBEIIb abundance than G4. The weak SBEIIb protein bands in G1 and G2 may be due to an effect of various levels of *SBEIIb* down-regulation, which occurred in the independent lines. The results of SDS-PAGE analysis on total GBPs were also consistent with protein band patterns from two parental lines (Fig. 3b). Also, the abundance of SSI and GBSSI in G1 and G2 was higher than that in G3 and G4. Three protein bands were also detected at 100–120 kDa in both G1 and G2 (Fig. 3b) as observed in A203 lane (Fig. 2).

Grain weight and morphology

There were no statistically significant differences in grain weight among four genotype groups ($p < 0.05$) (Table 1), although there were some variations of the grain weight among them. The range of grain weights was from 19.5 mg (G1) to 23.8 mg (G3).

Intact grains from four representative lines for the four genotype groups were examined by stereoscopic microscopy. Grain appearances were opaque for G1 and G2 groups, and translucent for G3 and G4 groups (Fig. 4). The grain shapes

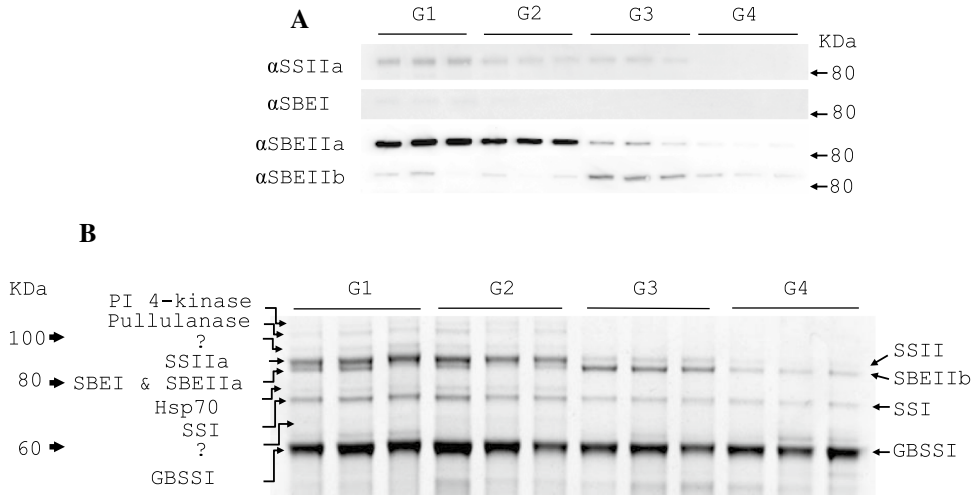

Fig. 3 Comparative analysis of starch granule bond proteins in mature rice grain starch of four genotype groups by immunoblot (**a**) and SDS-PAGE (**b**). Samples from three independent lines of each genotype group were used. The names of genotype groups are labeled on the top of each figure. G1: $SSIIa^{ind}SBEIIb^{DR}$, G2: $SSIIa^{jap}SBEIIb^{DR}$, G3: $SSIIa^{ind}SBEIIb^{WT}$, G4: $SSIIa^{jap}SBEIIb^{WT}$. Panel A: The protein bands detected by various antibodies are indicated on the left. The molecular sizes are labeled on the right of the gel in kDa. Panel B: The identity of each protein band is indicated by an arrowhead on both sides

Table 1 Grain weight, starch granule particle size, total starch content (TSC), apparent amylose content (AAC) and swelling power of rice grain starch in different genotype groups and controls

Group	Genotype		Grain weight (mg)	Small granules (%) < 1.9 μm	Small granules (%) < 10 μm	Small granules (%) < 20 μm	TSC (%)	AAC (%)	Swelling power
	SSIIa	*SBEIIb*							
G1	*ind*	DR	19.5 ± 0.2^{a}	7.3 ± 0.2^{b}	77.4 ± 3.2^{c}	94.7 ± 1.4^{b}	93.1 ± 1.1^{a}	28.4 ± 1.7^{a}	10.4 ± 0.3^{a}
G2	*jap*	DR	22.9 ± 0.1^{a}	7.5 ± 0.9^{b}	85.8 ± 0.4^{b}	96.8 ± 0.3^{b}	90.3 ± 1.3^{b}	27.7 ± 0.7^{a}	7.8 ± 1.2^{b}
G3	*ind*	WT	23.8 ± 0.4^{a}	9.7 ± 0.6^{a}	89.8 ± 0.7^{a}	100 ± 0^{a}	93.6 ± 1.6^{a}	15.1 ± 0.7^{b}	11.0 ± 1.3^{a}
G4	*jap*	WT	21.8 ± 0.2^{a}	10.5 ± 0.2^{a}	92.5 ± 0.8^{a}	100 ± 0^{a}	94.5 ± 1.3^{a}	12.5 ± 2.2^{b}	13.3 ± 1.0^{a}

Each value is the mean of three biological replicates. The mean values and standard variation values are shown as mean ± SD. The mean values within columns followed by different letters are significantly different at $p < 0.05$

ind indica; *jap* japonica; *DR* down-regulated; *WT* wildtype

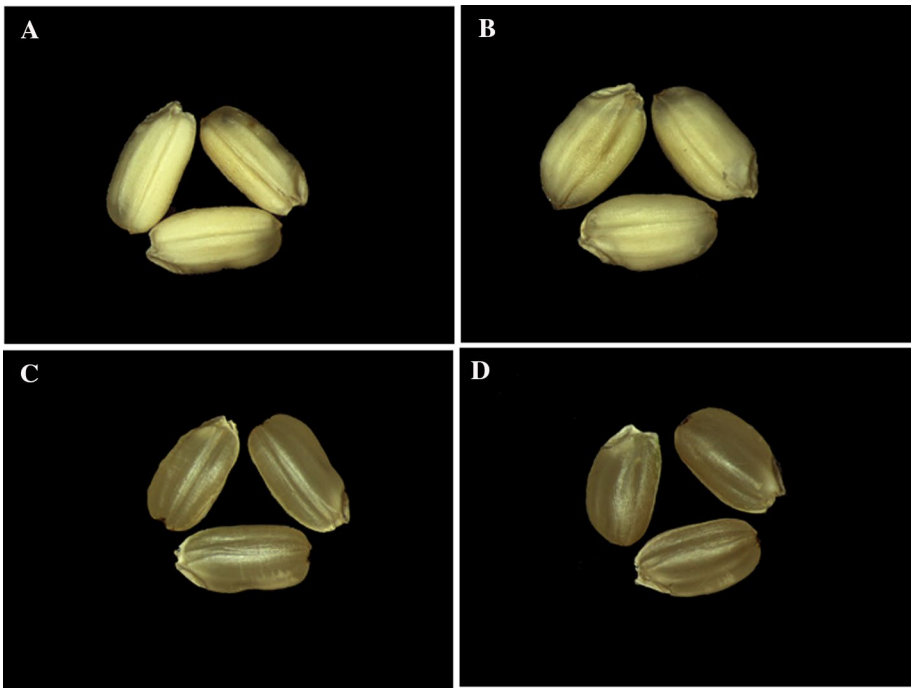

Fig. 4 Stereoscopic photographs of the morphology of rice grains. One line from each genotype was photographed. **a** G1 genotype group (*SSIIa*^{ind}*SBEIIb*^{DR}), **b** G2 genotype group (*SSIIa*^{jap}*SBEIIb*^{DR}), **c** G3 genotype group (*SSIIa*^{ind}*SBEIIb*^{WT}), **d** G4 genotype group (*SSIIa*^{jap}*SBEIIb*^{WT})

were also varied among four lines as one of parental line 3–12 originally was derived from the crosses an *indica* rice, IR64, and *japonica* rice, Nipponbare (Luo et al. 2015a).

Two types of rice grain morphology were observed among the four genotype groups. Polished grains of G1 and G2 lines were opaque throughout similar to the *SBEIIb* down-regulated parent, A203, while polished grains of G3 and G4 showed uniformly translucent endosperms similar to Nipponbare. No significant differences were observed either between G1 and G2 or between G3 and G4 (data not shown).

Starch granule phenotypic analyses

SEM analysis showed that starch of G1 and G2 genotype groups contained more round starch granules, some large round starch granules, and a few starch granules with polyhedral structure (Fig. 5a, b). After the isolation and purification process, large starch granules of G1 and G2 were structurally intact, while those of G3 and G4 were completely disassociated into polygonal granules. Compared with those angular typical rice starch granules in G3 and G4 (Fig. 5c, d), G1 and G2 exhibited non-angular and rounded starch granules. The edges of numerous starch granules in G1 and G2 became less apparent. There were a few very large granules in G1 and G2 genotype groups, the surface of which was irregularly undulating with protrusions and crack lines on the surface (Fig. 5a, b). The differences in starch granule appearance between G1 and G2 groups, and G3 and G4 groups were similar to those between starch granules from the parents (Nipponbare and A203) as shown in Fig. 5e, f. However, A203 did not have any large round starch granules.

Particle size analysis revealed that the percentage of starch granules sizes of purified starch was different in four genotype groups. The portion of three selected sizes of starch granules of G1 and G2 groups was significantly lower than those in G3 and G4 groups in the fractions (< 1.9 μm, < 10 μm and < 20 μm) ($p < 0.05$) (Table 1). Generally, the majority of starch granules were 1.9–10 μm in all four genotype groups (Fig. 6). G1 and G2 groups had around 3% fewer very small granules (< 1.9 μm) than that in G3 and G4 groups (Table 1). In < 10 μm fraction, the difference was profound among four groups with the order from less to more starch granules with the size at < 10 μm as G1 < G2 < G3 < G4. Approximately 3–5% of starch granules were over 20 μm in G1 and G2 genotype groups, whereas all starch granules in G3 and G4 groups were under 20 μm (Table 1).

Starch structural analyses

Variations in TSC and AAC were observed among four genotype groups of grains. All four genotype groups contained over 90% in TSC. The TSC of G1, G3, and G4 groups were comparable between 93 and 95% (Table 1). But, the G2 group having just over 90% TSC was significantly lower than the other three groups. The reduction of TSC was also observed with a 5% lower in A203 compared with its corresponding wildtype (Butardo et al. 2011). The AAC of G1 and G2 was comparable and remarkably higher than G3

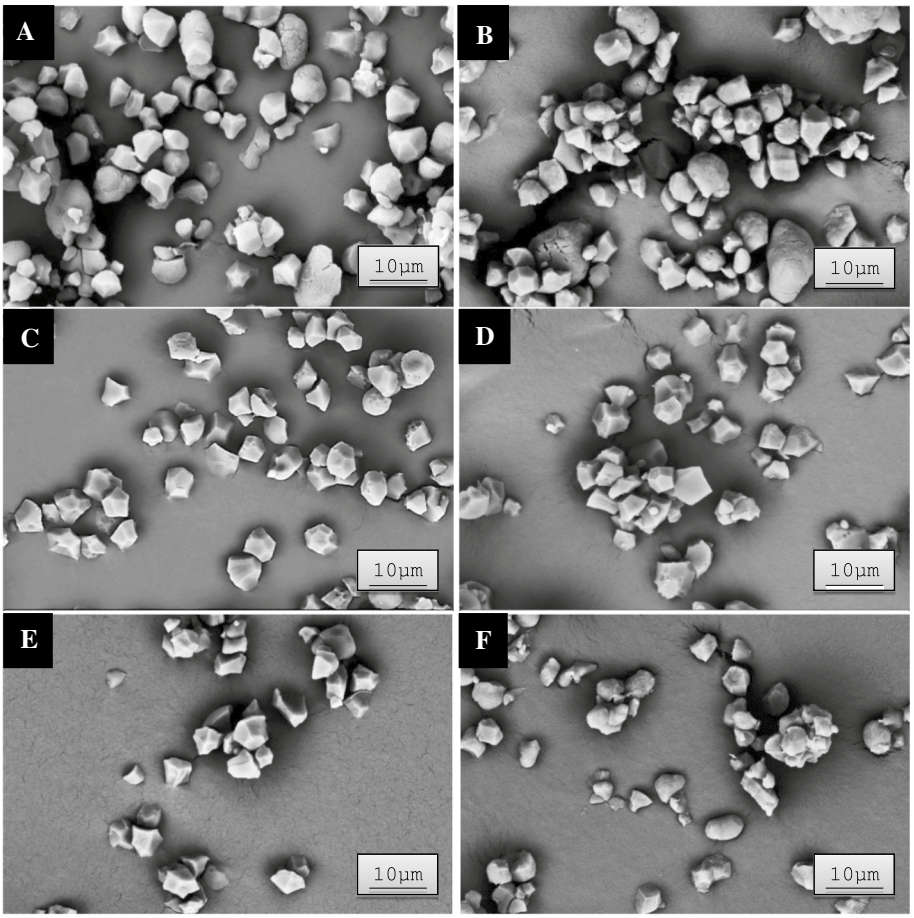

Fig. 5 Starch granule morphology analysis of purified rice starch of four genotype groups using SEM. The starch samples used for analysis are as follows: **a** G1 genotype group (*SSIIa*^{ind}*SBEIIb*^{DR}), **b** G2 genotype group (*SSIIa*^{jap}*SBEIIb*^{DR}), **c** G3 genotype group (*SSIIa*^{ind}*SBEIIb*^{WT}), **d** G4 genotype group (*SSIIa*^{jap}*SBEIIb*^{WT}), **e** Nipponbare, **f** ami-SBEIIb (A203). Bars on the low right corner of the photos represent 10 μm

and G4, which nearly doubled the figures of G3 and G4 (Table 1).

The SEC analysis of debranched starch revealed two types of starch chain length distribution patterns for the four genotype groups (Fig. 7). The proportion of amylose fraction (approximately 400–4000 DP) was comparable in all groups, while the difference appeared in the amylopectin fraction (about 4–400 DP). Compared with G3 and G4, G1 and G2 exhibited a marked decrease in 4–40 DP (peak 1) and an increase in chains of 40–400 DP (peak 2).

Further analysis of amylopectin chain length distribution (CLD) using FACE showed a peak change from DP 12 in G3 and G4 to DP 15 in the G1 and G2 (Fig. 8a). By the CLD difference plot, it was shown that the profiles of G1 and G2 were very similar with a remarkable decrease in DP 6–14 and an increase in DP 16–37 chains in comparison to G4 (Fig. 8b). G3 carried a different type of *SSIIa* to G4, showing a different profile with a decrease in DP 6–11 and an increase in DP 12–DP 23 (Fig. 8b).

Starch swelling power, gelatinization, and thermal properties

The starch swelling power test found that the four genotype groups had various capacities for holding water in their gelatinized starch. The order of the starch swelling power of the four groups was G4 > G3 > G1 > G2 (Table 1). Although the difference of the starch swelling power between G1, G3, and G4 was up to 3, it was not determined to be statistically significantly different ($p < 0.05$). Only G2 showed significantly lower swelling power than the other groups ($p < 0.05$). The difference between G2 and G4 groups was 5.5, while the G3 group was 2.3 lower than the G4 group.

The DSC analysis revealed the variation of starch thermal property among the four genotype groups. The amylopectin gelatinization temperatures (GTs) of G1 and G2 were higher than those of G3 and G4, respectively. In particular, the G4 was significantly lower than all the other groups for GTs ($p < 0.05$), whereas G1 and G2 were comparable (Table 2). Regarding the enthalpy of amylopectin gelatinization (ΔH1), G1 and G2 were at the same level, but G3 (1.7 J/g) was significantly lower compared with G4 (2.4 J/g) and the other two groups (over 2.5 J/g) ($p < 0.05$). The lipid-amylose complex dissociation, the onset, peak and end temperatures,

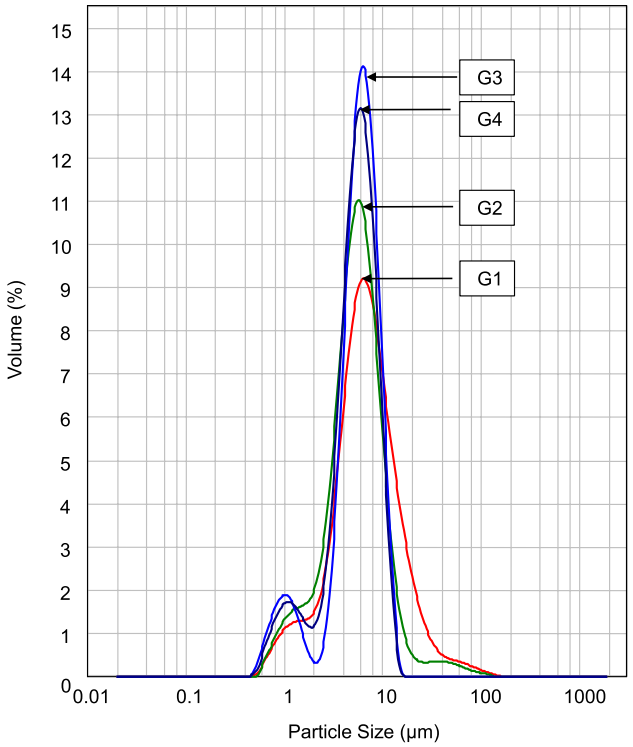

Fig. 6 Particle size analysis of grain starch granules of four rice genotype groups. The *y*-axis expresses the amount of each size of starch granules as a percentage of total starch in volume. The *x*-axis represents the size of starch granules. The different sample groups are labeled (color figure online)

respectively, were not significantly different among the four groups (Table 2). However, G1 and G3 exhibited a remarkable decrease in the dissociation enthalpy of amylose–lipid complexes (ΔH2) compared with G2 and G4, respectively (Table 2). The reduction in ΔH2 was also obtained when comparing G1 with G3 or G2 with G4, respectively, though the differences were not determined to be statistically significant.

Discussion

Pleiotropic effects of different SSIIa alleles on starch granule bound proteins in SBEIIb down-regulated starch in rice

The first objective of this work was to study the roles of the two *SSIIa* alleles on the deposition of starch biosynthetic enzymes within the starch granule of *SBEIIb* down-regulated rice grains. The downregulation of *SBEIIb* in line A203 caused a substantial change in the number of GBP compared to the parent Nipponbare. Aside from starch synthases (SSIIa, SSI, and GBSSI), which are typically present in Nipponbare, other proteins including PI-kinase, pullulanase, SBEI, SBEIIa, HSP70, and three uncharacterized proteins were additionally detectable in line A203. As starch granules were treated with proteinase K during starch preparation in this study, these additional proteins were present inside the starch granules. The result is supported by a recent report of *SBEIIb* inactive or null mutant with the same distribution pattern of the interior starch granule associated proteins in rice (Crofts et al. 2018). Two of the uncharacterized additional protein bands here were identified as SSIIIa (~ 250 kDa) and PPDK (pyruvate phosphate dikinase, ~ 100 kDa) in that study (Crofts et al. 2018). PI 4-kinase and HSP70 were reported indirectly involve in translocating, correctly folding, and solubilizing starch synthetic enzymes during import into the amyloplast (Yu et al. 1998), as well as in regulating physiological process

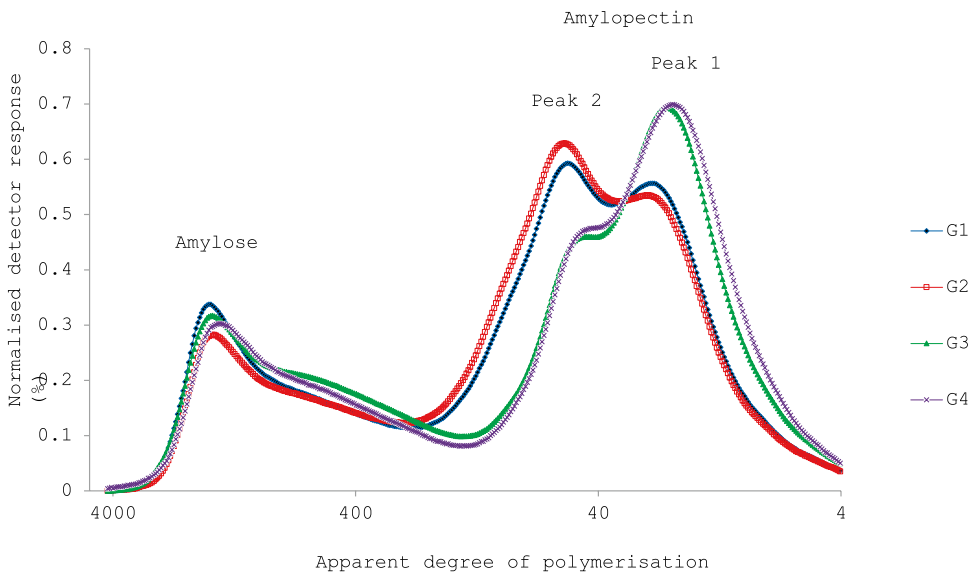

Fig. 7 Comparison analysis of debranched rice starch of four genotype groups using SEC. Distribution of normalized signals of debranched starch is plotted in the picture for each sample. Three independent lines of each group were used for data integration. The different samples are shown on the right. The two peaks of amylopectin and amylose are marked on the top (color figure online)

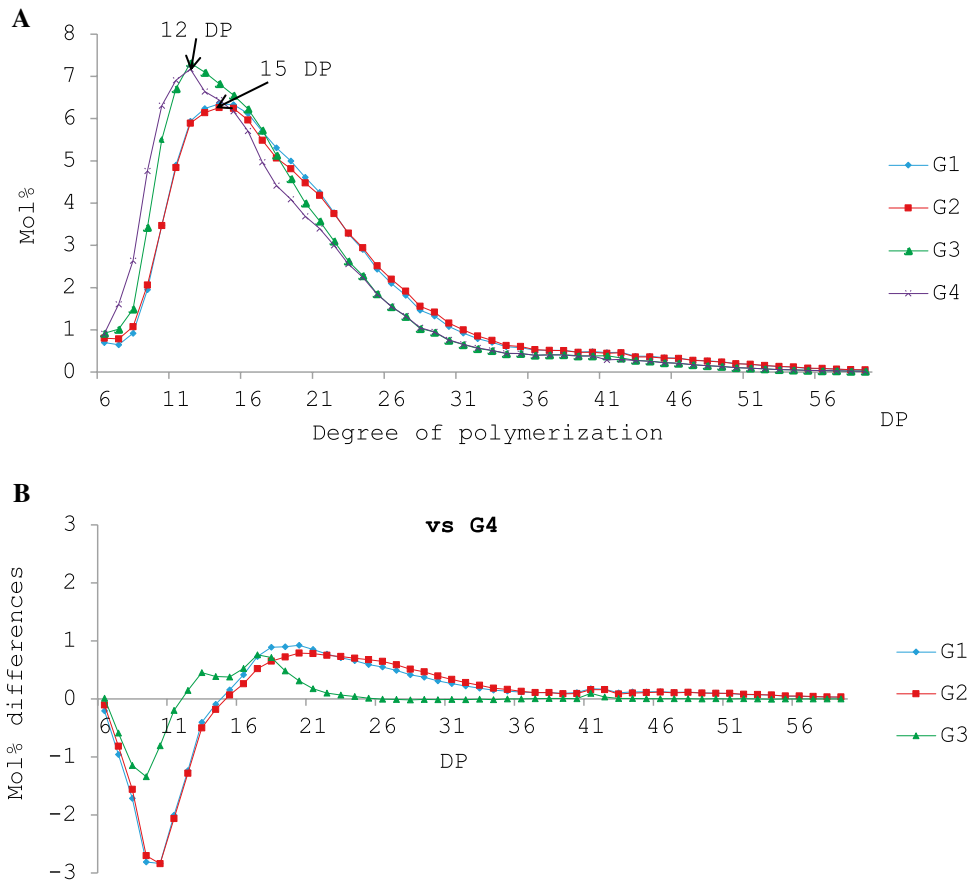

Fig. 8 Comparison of chain length distribution (CLD) of debranched starch of four genotype groups using CE. **a** shows the Mol% plots of CLD profiles of four different genotype groups and four genotypes, and two peaks are labeled on the top. **b** shows the Mol% difference of CLD profiles of G1, G2, and G3 genotype groups compared with the G4 genotype group. The indication of different samples is shown on the right (color figure online)

Table 2 Thermal characteristics of rice grain starch in different groups and controls

Group	Genotype		To1 (°C)	Tp1 (°C)	Te1 (°C)	ΔH1 (J/g)	To2 (°C)	Tp2 (°C)	Te2 (°C)	ΔH2 (J/g)
	SSIIa	*SBEIIb*								
G1	*ind*	DR	76.7 ± 1.9[a]	88.1 ± 0.5[a]	96.8 ± 0.5[a]	2.8 ± 0.2[a]	103.4 ± 0.5[a]	110.0 ± 0.5[a]	116.0 ± 1.5[a]	0.19 ± 0.1[b]
G2	*jap*	DR	77.5 ± 1.4[a]	87.8 ± 0.1[a]	96.3 ± 0.2[a]	2.7 ± 0.1[a]	102.4 ± 0.9[a]	107.0 ± 2.0[a]	114.6 ± 3.9[a]	0.52 ± 0.2[a]
G3	*ind*	WT	70.0 ± 3.6[a]	87.5 ± 0.6[a]	95.5 ± 0.3[a]	1.7 ± 0.1[b]	102.1 ± 0.9[a]	110.4 ± 0.3[a]	118.0 ± 0.9[a]	0.28 ± 0.0[b]
G4	*jap*	WT	65.5 ± 3.4[b]	81.0 ± 1.0[b]	89.6 ± 1.3[b]	2.4 ± 0.3[a]	102.2 ± 1.3[a]	108.0 ± 1.4[a]	115.4 ± 0.7[a]	0.72 ± 0.2[a]

To1, Tp1, and Te1 are onset, peak, and end gelatinization temperatures, and ΔH1 is the gelatinization enthalpy at the amylopectin gelatinization peak. To2, Tp2, and Te2 are onset, peak, and end dissociation temperatures of amylose–lipid complexes dissociation, and ΔH2 is the dissociation enthalpy at the dissociation peak of amylose–lipid complexes. Each value is the mean of three biological replicates, representing mean ± standard deviation. Mean values within the same column with different letters are significantly different at $p < 0.05$

ind indica; *jap* japonica; *DR* down-regulated; *WT* wildtype

(Ischebeck et al. 2010). HSP70s are important for protein translocation into amyloplast stroma (Yu et al. 1998), mitochondria and the endoplasmic reticulum (Su and Li 2010). SBEI and SSIIIa were previously reported as tightly granule-bound protein in developing endosperms of an *SBEIIb*-null rice mutant (Abe et al. 2014; Asai et al. 2014). Studies focused on *SBEIIb*-null mutant of maize discovered that SBEI, SBEIIa, and starch phosphorylase (SP) are associated with starch granules (Grimaud et al. 2008; Liu et al. 2009). It was suggested that SBEI, SBEIIa, and SP are included in protein complexes to compensate for the loss of the former component SBEIIb in the endosperms of the mutant. Thus, in its absence, the role of SBEIIb in amylopectin cluster synthesizing machinery is executed by SBEI, SBEIIa, and SP.

The starch synthetic enzyme complexes have been reported in rice, suggesting the mechanism of the functional protein complexes in developing endosperm is generally accepted for all cereals (Tetlow et al. 2004; Crofts et al. 2015). The deposition of SSIIIa, SBEI, SBEIIa, and pullulanase in *SBEIIb* down-regulated starches may be a

consequence of involving multiple amyloplast stromal protein complexes at different developmental stages of cereal endosperm when SBEIIb is absent or inactive inside starch granules. Nevertheless, the function of SBEIIb in *SBEIIb*^{DR} lines cannot be fully recovered by SBEI, and SBEIIa indicated by the lack of short chains of DP6 ~ 13 in amylopectin CLD. This is also supported by a more recent study, in which the impact of the loss of SBEIIb activity on starch structure and properties was most remarkably compared with SBEI and SBEIIa using mutants of multiple *SBE* genes in different combinations (Sawada et al. 2018). The increase of SSI and SSIIa abundance in *SBEIIb*^{DR} starch granules suggests the two enzymes are more extensively involved in enzyme complexes in *SBEIIb* down-regulated endosperms than wildtype. The additional amount of SSI and SSIIa may help the elongation of the short chains (DP < 6) formed by SBEIIa to reach DP ≤ 16 more efficiently to compensate for the lack of branches in this range in *SBEIIb* down-regulated starches.

As mentioned previously, SSIIa plays a central role in directing the association of other components of protein complexes to starch granules (Liu et al. 2012). The amount of SSI and SBEIIb in starch granules is correlated with that of granule-bound SSIIa which has been widely reported in cereals (Yamamori et al. 2000; Morell et al. 2003; Umemoto et al. 2004; Umemoto and Aoki 2005; Kosar-Hashemi et al. 2007; Grimaud et al. 2008; Luo et al. 2015b; Crofts et al. 2018). Our result here again shows such a pleiotropic effect in starches of *SSIIa*^{ind} and *SSIIa*^{jap} alleles in the *SBEIIb*^{WT} background but not in the *SBEIIb*^{DR} background. The abundance of SSIIa in starch granules is much higher in *SSIIa*^{ind}*SBEIIb*^{DR} than *SSIIa*^{jap}*SBEIIb*^{DR}, whereas the variation of SBEI, SBEIIa, and SSI are not so remarkable. This result leads to a hypothesis that SSIIa associates to starch granules possibly in two different forms during rice endosperm development: one is in monomer and/or oligomer form, the other is in the form of protein complex associating with other enzymes. Oligomerization of SSII was recently observed using bioinformatic and in vitro analyses of recombinant *Arabidopsis* SSII (Patterson et al. 2018). Although that study indicated that the formation of homodimers required the N-terminal region of the protein, which is highly variable among plant species, it still strongly supports the hypothesis in rice in this study. Based on this hypothesis, one explanation for the high abundance of SSIIa proteins in mature starch granules may be that the higher affinity of *indica* type SSIIa directed more SSIIa monomers and/or oligomers involved in starch biosynthesis and attached to starch granules in *SSIIa*^{ind}*SBEIIb*^{WT} than *SSIIa*^{jap}*SBEIIb*^{WT} endosperm. But the *indica* type SSIIa cannot direct more of those additional proteins in an equivalent additional amount of SSIIa protein to deposit in starch granules of *SBEIIb*^{DR} background. Therefore, the ability of *indica* type SSIIa may be comparable with *japonica* type SSIIa, in terms of protein-starch interaction, during starch synthesis in *SBEIIb*^{DR} background.

Effects of different SSIIa alleles on starch granule morphology, apparent amylose content and starch content of SBEIIb down-regulated starch in rice

SBEIIb^{DR} allele also affected the grain appearance to opaque in both *SSIIa*^{ind}*SBEIIb*^{DR} and *SSIIa*^{jap}*SBEIIb*^{DR} grains, as described in early publications (Tanaka et al. 2004; Butardo et al. 2011). The high expression of SSIIa in *SSIIa*^{ind} allele could not recover such opaque appearance of the rice grain in *SBEIIb*^{DR} grains.

The early publications showed that down-regulating SBEIIb in rice grain changes the higher level of organization of starch granules, forming compound starch granules in wildtype rice grain, which then changes the morphology of starch granules to spherical shape (Tanaka et al. 2004; Wei et al. 2010; Butardo et al. 2011). It was thought that the activity of SBEs affects the cluster size of amylopectin during endosperm development (Tetlow et al. 2004). Our results showed that a portion of starch granules in *SBEIIb*^{DR} background are larger in size based on our SEM analysis. This was also supported by the particle size distribution analysis, which revealed that about 5% of starch granules were over 20 μm in diameter in *SBEIIb*^{DR} starch. In contrast, *SBEIIb*^{WT} starch did not contain any large granules (> 20 μm). The bigger starch granules were present in both *SSIIa*^{ind}*SBEIIb*^{DR} and *SSIIa*^{jap}*SBEIIb*^{DR} grains, suggesting that *SSIIa*^{ind} cannot recover the phenotypic changes in starch granules induced by *SBEIIb* down-regulation.

In the current study, the association of increased AAC and altered CLD with reduced angular starch granule proportion was observed in *SBEIIb*^{DR} groups as previously reported on *SBEIIb* down-regulated lines of rice (Butardo et al. 2011). In comparison, the variation of AAC was not observed between *SSIIa*^{ind} and *SSIIa*^{jap} alleles in neither *SBEIIb*^{WT} nor *SBEIIb*^{DR} background, even though the CLD patterns were different. Such AAC results confirmed that the mutation in *SSIIa* does not affect amylose content. High AAC in G1 and G2 grains was derived from long-chain of amylopectin, not true amylose. In contrast to the mutation of *SSIIIa* in the *SSIIIa* and *SBEIIb* double-null mutant, the knockout of *SSIIIa* increased the amylose content of *SBEIIb* mutant to 45% from under 30% (Asai et al. 2014). It could be that SSIIIa possesses the major SS activity in the soluble fraction (Fujita et al. 2006, 2007). Although the SSs (SSI, SSIIa, and SSIIIa) share overlapping chain-length preferences, they cannot fully complement each other (Crofts et al. 2017). Knocking-out SSIIIa causes a remarkable reduction of amylopectin synthesis and increases the AAC level as a consequence.

SSIIa^{jap}*SBEIIb*^{DR} showed a reduction of about 4% TSC compared with *SSIIa*^{jap}*SBEIIb*^{WT} consistent with previous reports on *SBEIIb* knockout mutant EM10 (Abe et al. 2014; Asai et al. 2014). The decrease in TSC indicates that loss of SBEIIb or down-regulation of *SBEIIb* induces the decrease in starch biosynthesis due to a reduction in amylopectin and amylose biosynthesis as evidenced by SEC analysis and the unchanged amylose content. However, the TSCs of *SSIIa*^{ind}*SBEIIb*^{DR} was comparable with *SSIIa*^{ind}*SBEIIb*^{WT}, and higher than that of *SSIIa*^{jap}*SBEIIb*^{DR}, which indicated that *indica* type *SSIIa* could increase starch content in *SBEIIb*^{DR} background, including both amylose and amylopectin. However, the grain weight of *SSIIa*^{ind}*SBEIIb*^{DR} did not have a significant difference with that of *SSIIa*^{jap}*SBEIIb*^{DR}. As the availability of the grains from each line of four genotypes was the limitation for current study, the relationship between the increase of the starch content and no-change of grain weight for *SSIIa*^{ind}*SBEIIb*^{DR} could not be studied in detail. The mechanisms of the increase of starch content for the *indica* type *SSIIa* in *SBEIIb*^{DR} background remain to be further studied. The proportion of long amylopectin debranched chains (DP40–400) of *SSIIa*^{ind}*SBEIIb*^{DR} was slightly lower than *SSIIa*^{jap}*SBEIIb*^{DR} while that of short chains (DP 4–40) was marginally higher. The precise structural changes in amylopectin CLD were mild, with only a slight increase in DP16–21 and decrease in DP26–36 between *SSIIa*^{ind}*SBEIIb*^{DR} and *SSIIa*^{jap}*SBEIIb*^{DR}, which indicated that the increased abundance of SSIIa in *SBEIIb*^{DR} starch granules has only limited effect on amylopectin structure. These results suggest the combination of *SSIIa*^{ind} and *SBEIIb*^{DR} in rice only slightly modifies the amylopectin structure and stimulates starch accumulation in comparison to *SSIIa*^{jap} and *SBEIIb*^{DR} combination. An early publication also reported a similar effect of *SSIIa*^{ind} on the starch structure in rice grain containing the *SBEIIb*^{DR} and an indica allele of GBSSI (Itoh et al. 2017). Therefore, a more active isoform than the native SSIIa is required for efficient starch accumulation in rice when amylopectin biosynthesis has been significantly perturbed.

Effects of different SSIIa alleles on starch physical and thermal properties of SBEIIb down-regulated starch in rice

It was previously observed that *SBEIIb* knockout rice starch granules have more long glucan chains, due to the defect in amylopectin short chains, were more resistant to thermal gelatinization than wild type starch granules (Tanaka et al. 2004; Kubo et al. 2010). This variation in amylopectin CLD leads to high GTs and ΔH1 (Jane et al. 1999). Downregulation of *SBEIIb* generates more double-helical content and longer its length, which results in higher GTs and ΔH1 in rice (Dhital et al. 2015). In contrast, more short external chains of amylopectin lead to the weakening of the interaction among amylopectin molecules in crystal lamellae, which results in the low GTs and ΔH1 in wheat SSIIa mutant (Konik-Rose et al. 2007). As a more active form of SSIIa was present in *SSIIa*^{ind}*SBEIIb*^{DR} starch than *SSIIa*^{jap}*SBEIIb*^{DR}, it would be considered to be more efficient in synthesizing intermediate chains with DP13-24, and it may then affect the GTs and ΔH1. However, only subtle alterations in amylopectin structure groups were observed in the *SSIIa*^{ind}*SBEIIb*^{DR} starch with no variation of GTs and ΔH1 detected between *SSIIa*^{ind}*SBEIIb*^{DR} and *SSIIa*^{jap}*SBEIIb*^{DR}. Increased swelling power of *SSIIa*^{ind}*SBEIIb*^{DR} starch was found compared to that of *SSIIa*^{jap}*SBEIIb*^{DR}, which may be a result of the slight increase of the amylopectin intermediate chains (DP16 to DP21).

The variation of amylopectin CLD, shown in *SBEIIb*^{WT} vs. *SBEIIb*^{DR} and *SSIIa*^{ind} vs. *SSIIa*^{jap}, has an impact on the ΔH2 of amylose–lipid complexes. The current study showed that mean ΔH2 values were differentiated at 1/3 in both *SSIIa*^{jap}*SBEIIb*^{DR} vs. *SSIIa*^{jap}*SBEIIb*^{WT}, and *SSIIa*^{ind}*SBEIIb*^{DR} vs. *SSIIa*^{ind}*SBEIIb*^{WT}. Even though the differences were not statistically significant, the evidence probably indicated that the *SBEIIb* down-regulated starch had an increased proportion of the chain length of DP40–400, a reduced portion of DP4–40 in amylopectin (analysed by SEC), and a decreased amylose–lipid association enthalpy (ΔH2) of rice starch. With more intermediate chains (DP12 to 24) in amylopectin (analysed by CE) of *SSIIa*^{ind} starch than *SSIIa*^{jap}, ΔH2 value is significantly lower in *SSIIa*^{ind}*SBEIIb*^{WT} vs. *SSIIa*^{jap}*SBEIIb*^{WT}, and *SSIIa*^{ind}*SBEIIb*^{DR} vs. *SSIIa*^{jap}*SBEIIb*^{DR}. Therefore, these results showed that the change of ΔH2 values was mainly controlled by the *SSIIa* alleles with less contribution from SBEIIb alleles. The *SBEIIb*^{DR} *allele* reduced ΔH2 values in the *SSIIa*^{ind}*SBEIIb*^{DR} vs. *SSIIa*^{ind}*SBEIIb*^{WT}, and *SSIIa*^{jap}*SBEIIb*^{DR} vs. *SSIIa*^{jap}*SBEIIb*^{WT}, but they were not a significant difference in the population. The result was also consistent with the study in wheat, in which ΔH2 value is significantly increased when SSIIa is knocked out (Konik-Rose et al. 2007).

Implications for starch quality improvement in rice

Rice is a staple food in many developing countries and improving the nutritional quality of rice is an important research focus (Butardo and Sreenivasulu 2016), including the vital objective of raising the resistant starch (RS) level of rice grains (Butardo et al. 2011; Matsumoto et al. 2012). Increasing the intake of RS-rich food products benefits to human health by lowering the risk of cardiovascular disease and diabetes due to the characteristic of slow release of glucose, but also provides protection against colorectal cancer due to the high content of short-chain fatty acid produced from non-digestible starch fermentation (Topping and

Clifton 2001; Champ et al. 2003; Bird et al. 2010; Fuentes-Zaragoza et al. 2011; Higgins and Brown 2013). Starch with high amylose content is positively correlated with high RS (Ring et al. 1988; Faisant et al. 1993) due to the reduction of starch granule amylolysis (Dhital et al. 2015). Cooking and sensory qualities of rice, including starch textural properties, have major impacts on consumer preferences in different food cultures (Fitzgerald et al. 2009; Calingacion et al. 2014). Rice of high amylose content often has a firm texture, which is not preferred by consumers in many regions (Juliano 2001). The texture of cooked rice grain is related to not only amylose but the fine structure of amylopectin (Li et al. 2016). Improvement in the starch textural quality of rice, as well as increasing the RS content, will be a target in further breeding programs. Our study on *SSIIa* and *SBEIIb* interactions indicates that it is possible to improve rice grain quality, yet maintain their amylose content in *SBEIIb* down-regulation lines through manipulating the amylopectin chain length distribution. Genetic introduction of allelic variants of amylopectin biosynthetic genes is one of the applicable strategies.

Conclusions

Down-regulation of *SBEIIb* in rice increases the number of starch granule bound proteins, including PI 4-kinase, pullulanase, SBEI, SBEIIa, Hsp70, and three other unidentified proteins. The impacts of *SBEIIb* down-regulation in the *japonica* background on starch granule morphology, starch structure, and properties can be partly recovered by molecular breeding with an *indica SSIIa* allele. The active allele of *SSIIa* increases total starch content and starch swelling power, mildly modifies chain length distribution of amylopectin, and reduces the amylose–lipid complexes dissociation enthalpy of *SBEIIb* down-regulated starch without reducing the amylose content. This study sheds light on the improvement of the texture of rice grains with high resistant starch.

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Author contribution statement JL carried out the experiments and wrote the draft; QY finished the SDS-PAGE detection; CK did particle size and DSC analyses; MLC did mass spectrometry analysis; VMB contributed to experimental design; AM, SJ and ZL designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information file.

Compliance with ethical standards

Conflict of interest The authors declare that there are no competing interests.

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