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ORIGINAL PAPER

The different effects of starch synthase IIa mutations or variation on endosperm amylose content of barley, wheat and rice are determined by the distribution of starch synthase I and starch branching enzyme IIb between the starch granule and amyloplast stroma

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Abstract

Key message **The distribution of starch synthase I and starch branching enzyme IIb between the starch granule and amyloplast stroma plays an important role in determining endosperm amylose content of cereal grains.**

Abstract Starch synthase IIa (SSIIa) catalyses the polymerisation of intermediate length glucan chains of amylopectin in the endosperm of cereals. Mutations of *SSIIa* genes in barley and wheat and inactive SSIIa variant in rice induce similar effects on the starch structure and the amylose content, but the severity of the phenotypes is different. This study compared the levels of transcripts and partitioning of proteins of starch synthase I (SSI) and starch branching enzyme IIb (SBEIIb) inside and outside the starch granules in the developing endosperms of these *ssIIa* mutants and inactive SSIIa variant. Pleiotropic effects on starch granule-bound proteins suggested that the different effects of SSIIa mutations on endosperm amylose content of barley, wheat and rice are determined by the distribution of SSI and SBEIIb between the starch granule and amyloplast stroma in cereals. Regulation of starch synthesis in *ssIIa* mutants and inactive SSIIa variant may be at post-translational level or the altered amylopectin structure deprives the affinity of SSI and SBEIIb to amylopectin.

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Introduction

Starch synthase IIa (SSIIa) is a member of family of enzymes that catalyses the polymerization of glucan chains by transferring the glucosyl moiety of the soluble precursor ADP-Glucose to the reducing end of a pre-existing α-(1→4)-linked glucan primer (Fontaine et al. 1993). SSIIa in all cereals is involved in the synthesis of intermediate amylopectin chains with the degree of polymerisation (DP) 12–24 by elongating short chains (DP ≤ 10) of amylopectin. The reduction or complete loss of SSIIa can alter the composition and amount of starch in endosperms of barley (Morell et al. 2003), wheat (Yamamori et al. 2000; Konik-Rose et al. 2007), rice (Umemoto et al. 2002) and maize (Zhang et al. 2004). However, the resulting effects of the

different mutations in *SSIIa* on amylose content have not been comparatively studied in these major cereals.

Aside from SSIIa, granule-bound starch synthase I (GBSSI), SSI, SSIII and SSIV are the other members of the starch synthase family (See reviews from Ball and Morell 2003; James et al. 2003; Kossmann and Lloyd 2000; Rahman et al. 2000). GBSSI is the most abundant protein inside the starch granules and it is responsible not only for the synthesis of long amylose glucan chains with scarce branches (Ball et al. 1996), but also for the synthesis of long chains of amylopectin (Denyer et al. 1996; Maddelein et al. 1994). On the other hand, SSI, SSIIa and SSIII are involved in amylopectin synthesis and they work progressively on longer glucan chains during amylopectin elongation (Craig et al. 1998; Delvalle et al. 2005; Fujita et al. 2006, 2011; Konik-Rose et al. 2007; Jane et al. 1999; Li et al. 2011; Lin et al. 2011; Morell et al. 2003; Peng et al. 2001; Umemoto et al. 2002; Yamamori et al. 2000; Zhang et al. 2004). Last, SSIV appears to be involved in the initiation of starch granule formation based on studies in *Arabidopsis* leaves (Roldan et al. 2007; Szydlowski et al. 2009).

Starch branching enzymes (SBEs) are the other important class of enzymes involved in starch biosynthesis. They generate α-(1→6)-linkages on linear and branched glucan chains in the amylopectin molecule by simultaneous cleaving and transferring short α-(1→4) linked glucan chains to other chains within the starch molecule (Ball and Morell 2003; Rahman et al. 2000; Tetlow et al. 2004a). In cereals, SBEs are classified into two classes: SBEI and SBEII with the latter further classified into two isoforms, SBEIIa and SBEIIb. SBEIIb is the predominant branching enzyme in rice and maize (Gao et al. 1996; Garwood et al. 1976; Nishi et al. 2001). In contrast, SBEIIa appears to be the major branching enzyme in wheat endosperm (Regina et al. 2006), while both SBEIIa and SBEIIb appear to play an equal role in barley grain (Regina et al. 2010) in the determination of amylose content. Similar amounts of SBEIIa and SBEIIb were detected in the soluble fractions of both wheat and barley developing endosperms.

In cereal endosperms, SSs and SBEs are distributed inside and outside the starch granules in amyloplasts, termed as starch granule-bound proteins and soluble proteins, respectively (Denyer et al. 1993, 1995; Harn et al. 1998; Rahman et al. 1995; Tetlow et al. 2004a). Major starch granule-bound proteins of cereal endosperms are starch biosynthetic enzymes including GBSSI, SSI, SSIIa, SBEIIa and SBEIIb (Morell et al. 2003; Rahman et al. 1995). These enzymes are eventually trapped inside the semi-crystalline matrix of the starch granule during the course of starch biosynthesis (Denyer et al. 1993).

SSs and SBEs do not function independently during starch synthesis. For example, in maize, several studies have demonstrated the formation of multi-enzyme complexes of SSIIa, SBEIIa, SBEIIb and SSIII (Hennen-Bierwagen et al. 2008, 2009; Liu et al. 2009, 2012a; Tetlow et al. 2004b). Furthermore, SSI, SSIIa and SBEIIs were found to be involved in the assembly of one or more protein complexes in the amyloplasts of wild-type wheat and barley (Tetlow et al. 2004b, 2008; Ahmed et al. 2015).

SSIIa plays a crucial role in trafficking SSI and SBEIIb into the granule matrix through the formation of trimeric protein complexes (SSI-SSIIa-SBEIIb) and glucan-binding ability of SSIIa in maize (Liu et al. 2012b). The loss of the glucan-binding ability of SSIIa leads to the inability of SSI and SBEIIb in the protein complex to become granule-bound and to the reduction of functional SBEIIb (Liu et al. 2012b). The pleiotropic effect of SSIIa on other starch enzyme's catalytic activity (e.g. SBEIIb) is probably the reason why the loss/or reduction of SSIIa proteins in starch granules significantly changes the structural and functional properties of cereal starches. The phenotypic outcome of *SSIIa* mutation or variation include elevation in amylose content (AC), increase in the amount of short amylopectin chains resulting in the proportional decrease in the intermediate chains, reduction of amylopectin content, lowering of gelatinisation temperature (GT) and alterations in starch crystallinity and viscosity (Bao et al. 2009; Cuevas et al. 2010; Konik-Rose et al. 2007; Li et al. 2011; Morell et al. 2003; Umemoto et al. 2004, 2008; Zhang et al. 2004).

The SSIIa knockout mutant of barley (*sex6*) has high amylose content (~70 %) and novel grain phenotype due to the introduction of a stop codon mutation upstream of the catalytic region of the *SSIIa* gene (Morell et al. 2003). Previous studies on wheat SGP1 mutant showed the dosage effects of the *SSIIa* gene on amylopectin short chains and functional properties of wheat grains (Konik-Rose et al. 2007; Nakamura et al. 2005a; Yamamori et al. 2000). In rice, the research on the variations of SSIIa among different rice cultivars revealed that two of the SNPs that resulted in amino acid substitution in *SSIIa* between *japonica cv*. and *indica cv*. can alter the enzymatic activity and starch granule association ability of SSIIa which resulted in changes in starch properties (Bao et al. 2006; Luo et al. 2015; Umemoto and Aoki 2005; Waters et al. 2006).

This study focuses on comparing the association of starch biosynthetic enzymes to the starch granules of *ssIIa* mutants in barley, and wheat, and inactive SSIIa variant in rice. The changes in abundance of SSI, SBEIIa and SBEIIb due to the reduction or absence of SSIIa were quantified at RNA and protein levels, and the differences were compared among species. We provide solid evidence to support the hypothesis that SSIIa regulates starch synthesis through the redistribution of starch biosynthetic enzymes between the starch granules and stroma inside the amyloplast. This study corroborates the central role of SSIIa in the post-translational regulation by the formation of starch enzyme

complexes during starch biosynthesis in cereals as was also recently found in maize (Liu et al. 2012b).

Materials and methods

Plant materials

Plants were grown simultaneously at 15–25 °C for barley and wheat and at 27 °C for rice in glass houses at CSIRO Plant Industry (Canberra). Twenty to forty developing endosperms were collected at 15 days post anthesis (DPA) in tubes on dry ice and stored at −80 °C for the analyses of RNA, soluble proteins and starch granule-bound proteins as described below. Mature seeds were harvested for starch granule-bound proteins and starch property analyses. Homozygotes of mutant and wild-type *SSIIa* barley (*HvssIIa* and *HvSSIIa*, respectively) were screened from a backcross population of an *HvssIIa* mutant (Li et al. 2011). Mutant and wild-type wheat (*TassIIa* and *TaSSIIa*, respectively) were screened from a double haploid population from our previous work (Konik-Rose et al. 2007). A biparental population of rice was set up by crossing an *indica* (IR64) and a *japonica* cultivar (Nipponbare), to develop lines with *japonica* SSIIa (*OsssIIa*^{jap}) which had reduced SSIIa activities according to previous studies on rice (Umemoto and Aoki 2005) in an enzymatically active GBSSI background. Haplotypes of either *SSIIa* or *Wx* genes were genotyped in F2 generation with gene-specific DNA markers. Homozygotes of both *OsssIIa*^{jap} (inactive SSIIa variant) and *OsSSIIa*^{ind} (wild type) in *Wx*^{ind} background were selected from F3 generation (Luo et al. 2015). F5 selfing descendants were used for analyses in the study.

Genome DNA extraction and genotyping

Young leaves were freshly collected and used for genomic DNA extraction using Fast DNA Kit (BIO101 system, Q-BIO gene). Plants were genotyped by PCR to identify five lines for both *ssIIa* and *SSIIa* for all three cereals. For barley, PCR reaction was conducted using primers, ZLBSSIIaF and ZLBSSIIaR (Table S1), to select five lines of *HvssIIa* mutants (HH27, HH42, HH43, HH51 and HH84) and five lines of *HvSSIIa* wild types (HH21, HH60, HH61, HH110 and HH118). For wheat, PCR reaction was conducted using primers, ZLwSSIIaF and ZLwSSIIaR (Table S1), to select five lines of *TassIIa* mutants (A24, B22, B29, B63 and E24) and five lines of *TaSSIIa* wild types (A113, B32, B68, B70 and C9). For rice, two sets of cleavage amplified polymorphic sequences (CAPS) markers were used for the analysis of the *SSIIa* and *Wx* genes. For *SSIIa* genes, ZLRSSIIaGTF5 and ZLRSSIIaGTR5 were used for the amplification of PCR products, from which only the product with *indica* origin can be digested by *Mlu*I. For *Wx* gene, PCR products were amplified with ZLRWxGTF3 and ZLRwxGTR3 (Table S1), in which only the *Wx*^{jap} fragments were digestible by a restriction enzyme *Nhe*I. *OsssIIa*^{jap} lines carrying *japonica SSIIa* alleles (6–1, 6–7, 6–14, 6–19 and 15–1) and *OsSSIIa*^{ind} lines carrying *indica SSIIa* alleles (6–9, 6–20, 6–24, 5–9 and 14–12) were selected with *Wx* gene derived from *indica* rice in the F3 generation.

For each PCR (20 µl), 50 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.125 mM of each dNTP, 10 pmol of primers, 0.5 M glycine betaine, 1 µl of dimethylsulphoxide (DMSO) and 1.5 U of Advantage 2 Taq polymerase mix (Clontech) were used. The PCRs were conducted using a HYBAID PCR Express (Integrated Sciences) with one cycle of 95 °C for 5 min, 35 cycles of 94 °C for 45 s, 59 °C for 30 s and 72 °C for 3 min, one cycle of 72 °C for 10 min and one cycle of 25 °C for 1 min.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from endosperms at 15 DPA was extracted using NucleoSpin® RNA Plant Kit (Macherey–Nagel) and quantified using Nanodrop 1000 (Thermo Scientific). A total of 0.5 µg of RNA templates was used for the cDNA synthesis in a 50-µl reaction with ramp at 50 °C using SuperScript III reverse transcriptase (Invitrogen). The cDNA template (100 ng) was used in a 10 µl qRT-PCR reaction with the annealing temperature at 58 °C using previously-published RT-PCR primers (Table S2). The same pair of primers for the *Tubulin* gene was designed for three cereals which located at the highly homologous sequences in the exons at 3′ ends of the genes. However, the amplification was not suitable for *Tubulin* in rice; thus a different set of primers were used as published (Toyota et al. 2006). The amplification was conducted in a Rotor-Gene 6000 (Corbett) using Rotor-Gene™ SYBR® Green PCR Kit (QIAGEN). Comparative quantitation was analysed using *Tubulin* as a reference gene in the Real Time Rotary Analyzer Software (Corbett). For each sample, triplicates of qRT-PCR reactions were performed.

Isolation of soluble and starch granule-bound proteins from developing endosperm

Developing endosperms from kernels harvested at 15 DPA were homogenised and suspended in pre-chilled soluble protein extraction buffer at 1.5 µl/mg (0.25 M K_2HPO_4, pH7.5, 0.05 M EDTA, 20 % glycerol, Sigma protease inhibitor cocktail and 0.5 M DTT). The homogenate was centrifuged at 16,000*g* for 15 min at 4 °C. The supernatant containing soluble proteins was used for the estimation of protein concentration using Coomassie Plus Protein Assay

Reagent (Bio-Rad). Samples were stored at −20 °C prior to analysis. The pellet was kept for the starch preparation below.

The pellets kept from the soluble protein preparation were directly treated with proteinase K after washing with water, and then starch was purified as the procedures below. The Starch granule-bound proteins were prepared from purified starch according to Rahman et al. (1995) with minor alterations. Starch granules were boiled for 5–10 min in protein denaturing extraction buffer (50 mM Tris buffer, pH 6.8, 10 % glycerol, 5 % SDS, 5 % β-mercaptoethanol and bromophenol blue) at a ratio of 15 µl/mg starch. After centrifugation at 13,000*g* for 20 min, the supernatant was used for SDS-PAGE analysis.

Isolation of starch and extraction of starch granule-bound proteins from mature grains

Whole grains (approximately 100–150 mg) of each line from three cereals were ground in a ball bearing machine at a speed of 30 rpm for 30 s using WIG-L-BUG Mixer MSD (USA). Wholemeal products were first treated with 0.005 % NaOH, filtered through 0.5 mm nylon sieves, washed with water three times and then incubated with 0.5 mg proteinase K in 1 ml of 50 mM phosphate buffer at 37 °C for 2 h. The starch pellets obtained by centrifugation at 5000*g* were suspended and washed with water for three times following with centrifugations after each wash. After washing with acetone, the residual starches were air-dried at 37 °C overnight. Preparation of starch granule-bound proteins from mature grain starches were the same as that described earlier for developing endosperm starches.

SDS-PAGE and gel staining

For the quantitation of protein contents in starch, an equal amount of starches (4 mg) was used for the extraction of starch granule-bound proteins. Same volume of supernatant containing total proteins was loaded for each sample into NuPAGE Novex 4–12 % Bis–Tris Gels (Life technologies). This allows for the detection of variations in protein-binding patterns from the same amount of starches. Samples containing 20 µg total proteins were used for soluble proteins. The SDS-PAGE gels were run and detected as previously described (Butardo et al. 2012).

Immunoblotting

Anti-serum against GBSSI, SSI, SSIIa, SBEIIa and SBEIIb from previous studies are listed and enumerated in Table S3, including their antigen sources and specificities. Western blotting and detection was carried out as previously described (Butardo et al. 2012) using the same protein standards as above.

Quantitation of protein bands on SDS-PAGE gels and immunoblots

To quantitate and compare the abundance of proteins between different genotypes, two protein bands (80 kDa and 60 kDa) in 5 µL MagicMark^{TM} XP protein ladders (Invitrogen) were used as references. After visualising the protein bands, SDS-PAGE gels and immunoblots were scanned (Epson Perfection 2450 PHOTO; Epson America Inc., CA, USA) to image files for band intensity analysis using the Quantity One software package following the prescribed methods (Bio-Rad). Band 80 kDa was used for the quantitation of SBEIIa, SBEIIb and SSIIa, band 60 kDa for SSI and GBSSI.

Mass spectrometry

In-gel proteolytic digestion was done on selected protein bands from Coomassie Blue stained SDS-PAGE gels. Ion trap tandem MS was conducted as described by Butardo et al. (2012). Proteins were identified by the correlation of uninterpreted MS to entries in SwissProt/TREMBL, through ProteinLynx Global Server (Version 1, Micromass) (Colgrave et al. 2013).

Amylose content and starch chain length distribution

The amylose contents of samples were determined by analysing debranched starches using size-exclusion chromatography (SEC) as described previously (Butardo et al. 2012; Castro et al. 2005). Pullulan standards (Shodex P-82) calibrated with the Mark–Houwink–Sakaruda equation were used for the estimation of the molecular weight from the elution time. Samples were prepared and analysed in triplicate.

Determination of the chain length distribution of amylopectin was conducted by fluorescence-activated capillary electrophoresis (FACE), and samples were prepared as previously described (O'Shea and Morell 1996).

Statistical analyses

All data were subjected to statistical analyses (*t* test and one-way ANOVA, with Tukey post-test) using GraphPad Prism Version 5.01. Error bars represent standard error of mean (SEM). The statistical significance was defined at $P < 0.05$ and $P < 0.01$.

Results

Analysis of mRNA expression of selected starch synthetic genes

Results of gene profiling by quantitative reverse transcription PCR (qRT-PCR) revealed that level of *SSIIa* expression in homozygous mutant barley (*HvssIIa*) and wheat (*TassIIa*) endosperm was significantly lower than that of their corresponding wild-type lines ($p < 0.01$) (Fig. 1a, b). The relative expression of *SSIIa* in *HvssIIa* and *TassIIa* was approximately ~12 % of wild-type barley (*HvSSIIa*) and ~8 % of wild-type wheat (*TaSSIIa*) (Fig. 1a, b). The sharp reduction in *SSIIa* transcripts did not significantly affect the relative expression of *SSI, SBEIIa* and *SBEIIb* transcripts in barley and wheat mutants compared to their corresponding wild types (Fig. 1a, b). In contrast, the *SSIIa* expression between rice $OsssIIa^{jap}$ and $OsSSIIa^{ind}$ showed non-significant differences (Fig. 1c). This was also true for the expression levels of *SBEI, SBEIIa* and *SBEIIb* in rice (Fig. 1c).

Analyses of the abundance of granule-bound proteins in the starch of mature grain

To examine the relation between the protein band intensity on the protein gel and the amount of proteins loaded on gel, one to four mg of starch from *TassIIa* mutant and *TaSSIIa* wild type was used for the extraction of starch granule-bound proteins. Those proteins were separated on a protein gel (Fig S1), from which four starch granule-bound proteins with molecular weight 60 kDa and above were observed in *TaSSIIa*. In *TassIIa* mutant, GBSSI proteins were in band ~60 kDa. Protein bands were quantitated and the data were used for producing figures, the efficiency and R^2 values in Excel 2007. Quantitated protein bands showed a positive correlation between the protein band intensity and the amount of starch used for protein extraction for SSIIa, SBEII and SSI in *TaSSIIa* and GBSSI in *TassIIa* mutant (Figs. S2, S3). For *TaSSIIa* GBSSI, the protein band intensity was saturated when proteins were extracted from more than 2 mg starch. Due to the low levels of SSIIa, SBEII and SSI inside the starch granules, four mg starch was selected for extracting starch granule-bound proteins for the further analyses.

Three major granule-bound proteins with a molecular weight of 60 kDa or higher were detected in Sypro-stained gels for all the starch of mature grain of wild-type barley (Fig. 2a). They were identified from immunoblots as SSIIa/SBEIIa/SBEIIb in band ~85 kDa, SSI in band ~75 kDa and GBSSI in band ~60 kDa (Fig S4). In contrast, four starch granule-bound proteins with molecular weight 60 kDa and above were observed in samples of wild-type wheat and rice (Fig. 2b, c, respectively). They were further identified as SSIIa (~88 kDa), SBEIIa and SBEIIb (~83 kDa), SSI (~75 kDa) and GBSSI (~60 kDa) by immunonblot analyses (Figs. S5, S6, respectively). SBEIIa and SBEIIb migrated similarly, but the abundance of SBEIIb inside the starch granules was much higher than SBEIIa in all three cereals (Figs. S4, S5 and S6). The assignment of these proteins was also confirmed in barley and rice by MS (Table S4).

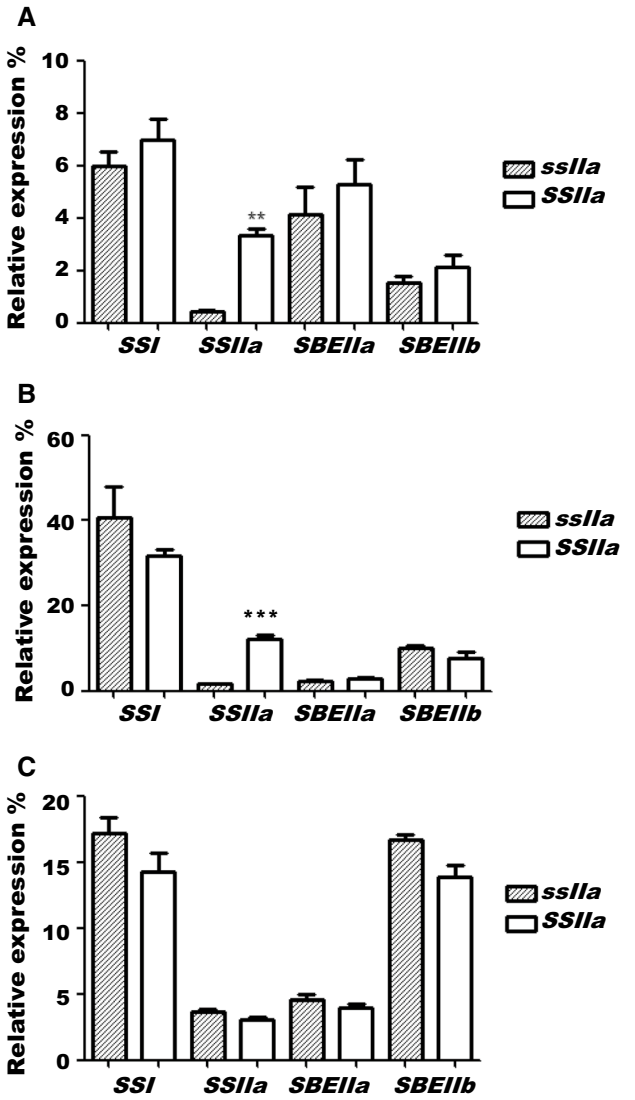

Fig. 1 Relative mRNA expressions of *SSI*, *SSIIa*, *SBEIIa* and *SBEIIb* in the endosperm at 15 DPA between *ssIIa* and *SSIIa* in barley (**a**), wheat (**b**) and rice (**c**) by quantitative RT-PCR. Total RNAs were used from five lines of each mutant and wild-type line of the three cereals. Triplicate assays were performed for each sample. *Tubulin* was used as a reference gene for each cereal. Names of genes are labelled *underneath bars*. The mutant and wild type of barley, wheat and rice are indicated on the *right* side of each figure. *Bars* with ** and *** show significantly difference at $p < 0.01$ and $p < 0.001$, respectively. *Error bars* indicate SEM

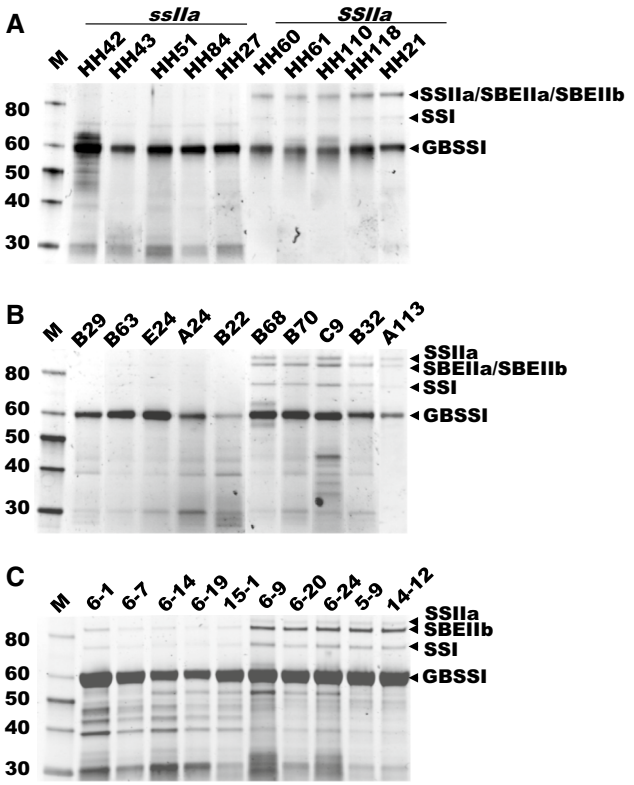

Fig. 2 Analysis of starch granule-bound proteins in mature grains between *ssIIa* and *SSIIa* of three cereals by SDS-PAGE followed by Sypro staining. SSIIa protein bands were absent from the mutants. The distributions of SBEIIa, SBEIIb and SSI inside starch granules of all *ssIIa* mutants were significantly lower than those of the wild types in barley (**a**), wheat (**b**) and rice (**c**). The *arrows* on the *right* indicate the positions of corresponding proteins. The sizes of molecular weight marker (M) were labelled in kDa

No SSIIa, SBEIIa, SBEIIb and SSI protein were detectable in *HvssIIa* mutant bands (Figs. 2a, 3a, S4). In the *TassIIa* mutant, SSIIa and SBEIIa were also not detectable, but SSI and SBEIIb were detected by immunoblot analyses although their abundance was so low that accurate measurements could not be made (Figs. 2b, 3a, S5). Similarly, only small amounts of SSIIa were found in rice inside the starch granules of *OsssIIa*^{jap} by immunoblot analyses (Figs. 2c, 3a, S6), and again its concentration was too low for a reliable quantitation (Fig. 4a). The quantity of SBEIIb and SSI in *OsssIIa*^{jap} was about ~25 and ~50 % compared to *OsSSIIa*^{ind} (Figs. 3a, S6).

Analyses of the protein abundance of starch biosynthetic enzymes in the soluble stroma and inside the starch granules of developing endosperm

To determine whether the low concentration of SSI, SBEIIa and SBEIIb inside the starch granules in the mature grains was due to the lower synthesis when the amount of SSIIa was reduced during grain development, protein bands for SSI, SBEIIa and SBEIIb were quantitated from all experimental lines in soluble fraction and inside the starch granules of the developing endosperms at 15 DPA.

The immunodetected protein bands in the soluble proteins of developing endosperms showed that the amount of both SBEIIb and SSI in *ssIIa* mutants and inactive SSIIa variant was significantly higher than that in *SSIIa* wild types in the three cereals (Figs. 3b, 4). The expression of SBEIIa was comparable between *ssIIa* mutants or inactive SSIIa variant and *SSIIa* wild types. A higher concentration of SBEIIa in the soluble fraction of amyloplasts compared to SBEIIb was detected in wheat and rice, while they have comparable amount in barley (Fig. 4b). No specific SSIIa protein band was identified in soluble proteins by immunoblot analyses (data not shown) which was possibly due to the presence of extensive interfering proteins in the soluble fraction.

In term of starch granule-bound proteins, neither barley *HvssIIa* nor wheat *TassIIa* lines showed the presence of SSIIa proteins in developing endosperm stage by immunoblot analyses (Figs. 3c, 5a, b). SBEIIb was decreased to less than ~5 % in mutant barley starch granules (Figs. 5a, S7), while the abundance of SBEIIb in mutant wheat decreased to ~25 % compared to the wild type (Figs. 3c, 5b, S7). The quantity of SSI protein inside starch granules of *HvssIIa* and *TassIIa* reduced to approximately ~25 % of their wild-type levels (Figs. 3c, 5a, b). However, while SSIIa in *OsssIIa*^{jap} was barely detectable as above (Figs. 3c, 5c), the levels of SBEIIb and SSI in *OsssIIa*^{jap} samples decreased to ~30 % and ~70 % of that in *OsSSIIa*^{ind} (Figs. 3c, 5c, S7). Similar patterns of reductions in the amount of starch granule-bound proteins were observed in the mature endosperms. However, on a per starch basis, the concentration of starch biosynthetic enzymes in starch granule-bound proteins of developing endosperms was higher than that in mature grains as a result of the dilution effect of higher starch levels in mature grain endosperm.

Mass spectrometric analyses of granule-bound proteins in mature grains of barley and rice

MS analysis of granule-bound proteins in mature grains was done to confirm the results of the Western blot analyses. In barley, peptide fragments for SSIIa, SBEIIa and SBEIIb were identified in HH21 (*HvSSIIa*) from protein band ~ 85 kDa by in-gel tryptic and chymotryptic digestion followed by LC-MALDI MS, with 64.2, 42 and 40.2 % sequence coverage, respectively (Table S4). Soluble protein samples were also analysed with mass spectrometry; however, the sequence coverage of peptide fragments was very low and the detection was interfered by the presence of a number of unrelated proteins in this study (data not shown).

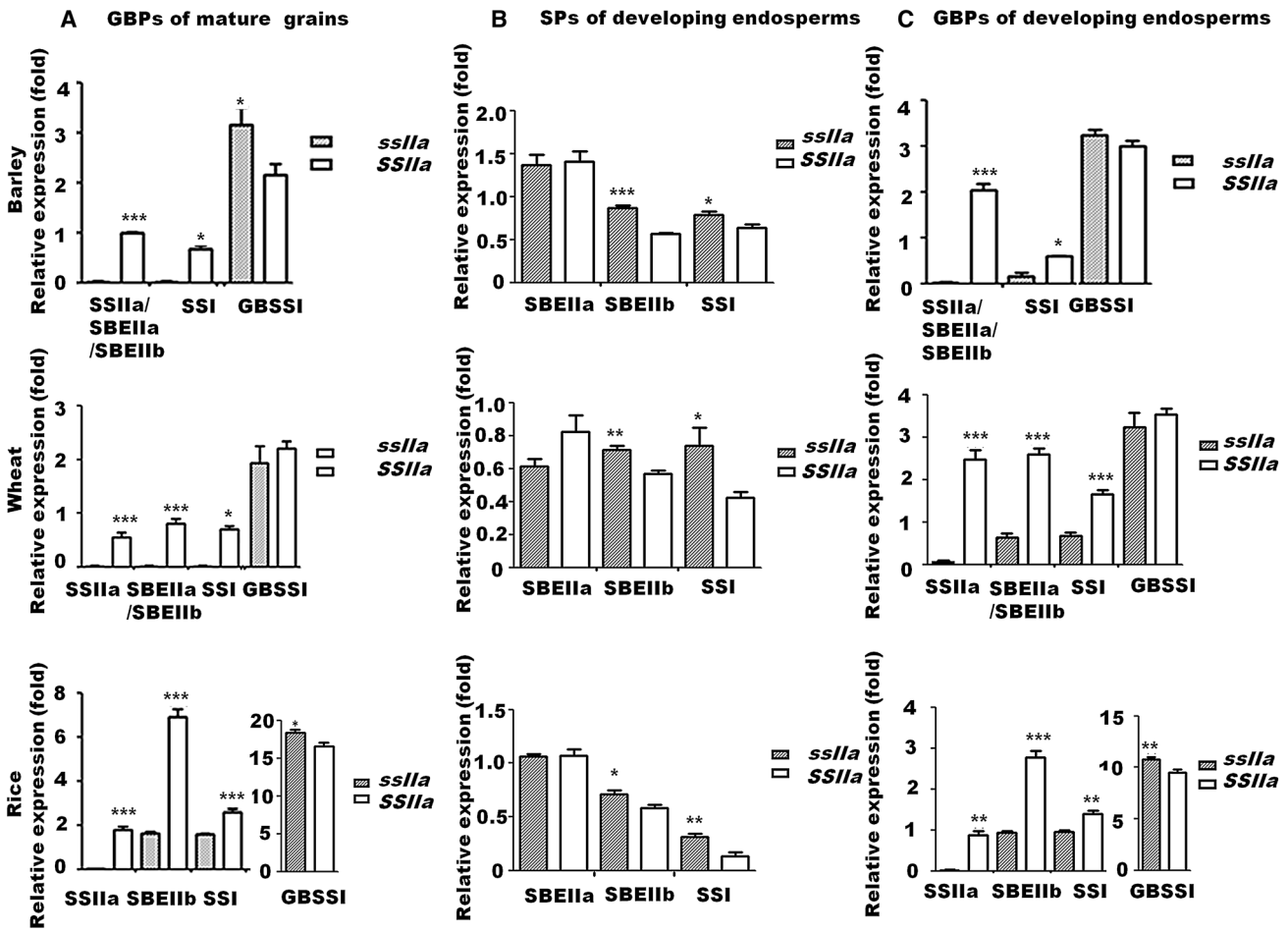

Fig. 3 Quantification analysis of selected proteins in mature grains and developing endosperms at 15 DPA of three cereals. *Bars* indicate the quantities of corresponding protein bands in Figs. 2, 4 and 5. Protein marker band 80 kDa was used as a reference band (its protein amount refers as 1) for the quantification of SSIIa, SBEIIa and SBEIIb, marker band 75 kDa for SSI and that of 60 kDa for GBSSI. **a** Starch granule-bound proteins in mature grains. **b** Soluble proteins in developing endosperms at 15 DPA. Image files obtained by scanning immunoblots of SPs were used. **c** Starch granule-bound proteins in developing endosperms at 15 DPA. The origins of proteins are labelled on the top. Names of protein bands are labelled *underneath bars*; mutants and wild type are labelled on the *right* side; cereal names are on the *left* side of the figures. For statistical analysis, five *lines* for mutants and wild type were used. *Bars* with *, ** and *** show significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

In rice IR64 (*OsSSIIa*), peptide fragments detected in band ~88 kDa SSIIa protein with 42.8 % of the sequence coverage. The ~83 kDa band was identified as SBEIIb with the sequence coverage of 24.9 %. GBSSI peptide fragments covering 55.5 % of the sequence were detected from the ~60 kDa band.

The results above were consistent with those of immunoblot analyses for GBSSI, SSIIa, SBEIIa and SBEIIb in different cereal samples (Table S4; Figs. S4, S6).

Structure of debranched starch from mature grains

In mature grains of *ssIIa* mutants and inactive SSIIa variant, the short chains (DP 6–10) of starch were increased and intermediate chains (DP 11–24) were decreased in all three cereals compared with corresponding wild types (Fig. S8). This result is consistent with those of previous studies in barley (Morell et al. 2003), wheat (Konik-Rose et al. 2007) and rice (Umemoto et al. 2002, 2008). Although the plot of the three cereals followed the same trend has been published elsewhere, we present here for the first time an inter-comparison of the differences in the chain length distribution among the three cereals (Table 1). The order of proportions of increase in the short chains in the two mutant cereals and inactive SSIIa variant rice was barley (9.3 %) > wheat (6.7 %) > rice (5.2 %). The decrease in the intermediate chains was barley (9.5 %) > wheat (8.8 %) > rice (6.8 %).

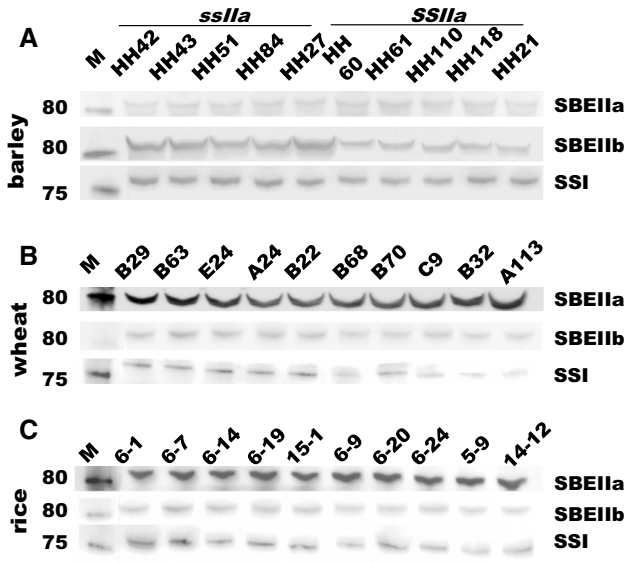

Fig. 4 Immunodetection analysis of soluble proteins in developing endosperms at 15 DPA between *ssIIa* and *SSIIa* of three cereals. Distributions of SBEIIa, SBEIIb and SSI in the soluble phase of all *ssIIa* mutants or inactive SSIIa variant and *SSIIa* wild-type lines in barley (**a**), wheat (**b**) and rice (**c**) were quantified by the intensity of marker band (M) on the left side in each picture. Names of proteins are indicated on the *right*, and the genotype and name of each line are labelled *above the lanes*

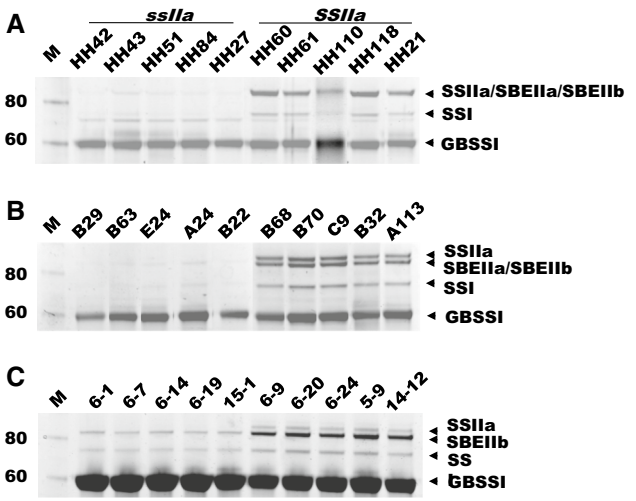

Fig. 5 Analysis of starch granule-bound proteins in developing endosperms at 15 DPA between *ssIIa* and *SSIIa of* three cereals by SDS-PAGE followed by Sypro staining. SSIIa protein bands were absent from *ssIIa* mutants or inactive SSIIa variant, while the distributions of SBEIIa, SBEIIb and SSI inside the starch granules of all *ssIIa* mutants or inactive SSIIa variant were significantly lower than those of the wild-type lines in barley (**a**), wheat (**b**) and rice (**c**), respectively. *Arrows* indicate the position of corresponding proteins. Names of lines and genotypes are given *above lanes*. The data of TaSSIIa are almost completely overlapped with the data of OsSSIIa, which is not easily viewed

Analysis of amylose content (AC)

The results of AC of mutants or inactive SSIIa variant and wild type used in the study are shown in Fig. 6 and Table 1. Significant increases in AC of *ssIIa* mutants were seen compared with their wild type confirming previous reports in *HvssIIa* (Morell et al. 2003) and *TassIIa* (Konik-Rose et al. 2007; Nakamura et al. 2005a; Yamamori et al. 2000). In contrast, the AC of the starch in $OsssIIa^{jap}$ was comparable to $OsSSII^{ind}$ with only slight elevation of about 0.9 % as published before (Bao et al. 2006; Luo et al. 2015; Umemoto and Aoki 2005). The relative order of increases of AC in mutant and inactive SSIIa variant compared with corresponding wild type in each species was barley (34.4 %) > wheat (17 %) > rice (0.9 %). When the percentage of elevated AC was calculated based on AC of wild type, the greatest elevation in the mutants and inactive SSIIa variant was 119 % in barley, and it was 64.1 and 4.5 % in wheat and rice, respectively.

Discussion

Starch synthase IIa (SSIIa) performs a critical function in starch biosynthesis of cereal grains, elongating the short chains of amylopectin so that proper packing of these chains occurs in the crystallisation clusters of amylopectin in the starch granule. However, mutants and inactive SSIIa variant of SSIIa in barley, wheat and rice have quite different phenotypes with barley having much higher amylose content than wheat and in rice the amylose content is hardly changed. This study sought to determine possible reasons for these differences by examining the expression of starch biosynthetic enzymes in developing and mature grains from wild type and mutants or inactive SSIIa variant as well as the distribution of the proteins between the granule and stroma within the amyloplast.

The expression and activity of SSIIa in cereal endosperms appears to be low compared with other SSs as shown by soluble SS activity measurement in vitro (Cao et al. 2000; Li et al. 1999). However, the effects of SSIIa deficiency are pronounced including pleiotropic effects on proteins inside the starch granules and structural alteration of amylopectin (Morell et al. 2003; Yamamori et al. 2000; Zhang et al. 2004). Indeed, Zhang et al. (2004) in their study on the sugary-2 mutant of maize suggested that the relative activity of the SSIIa isoform in the soluble phase is not indicative of the importance of this enzyme in amylopectin synthesis. Two possible explanations were proposed: one was that the SSIIa activity measurable in vitro was far more than that required in vivo; and the other was that the

Table 1 Starch properties of mature grains

Cereal	Genotype	CLD		AC (%)	CLD difference		AC difference (%)	AC difference (%)/WT AC (%)
		SCP (%)	ICP (%)		SCP (%)	ICP (%)		
Barley	HvssIIa	21.0 ± 0.2^{a}	57.1 ± 0.4^{b}	63.3 ± 1.2^{a}	9.3 ± 0.1^{a}	9.5 ± 0.3^{a}	34.4 ± 2.6^{a}	119
	HvSSIIa	11.8 ± 0.2^{b}	66.5 ± 0.7^{a}	28.9 ± 2.7^{b}				
Wheat	TassIIa	20.2 ± 0.2^{a}	55.7 ± 0.6^{b}	43.5 ± 3.2^{a}	6.7 ± 0.3^{b}	8.8 ± 0.5^{a}	17.0 ± 3.1^{b}	64.1
	TaSSIIa	13.5 ± 0.1^{b}	64.5 ± 0.7^{a}	26.5 ± 1.4^{b}				
Rice	OsssIIa^{jap}	15.8 ± 0.1^{a}	62.3 ± 0.4^{b}	20.9 ± 1.6^{a}	5.2 ± 0.1^{c}	6.8 ± 0.3^{b}	0.9 ± 0.9^{c}	4.5
	OsSSIIa^{ind}	10.5 ± 0.1^{b}	69.1 ± 0.1^{a}	20.0 ± 0.6^{a}				

For CLD and AC (%), mean values between *ssIIa* mutant/variant and wild type for each cereal with different letters are significantly different at $p < 0.01$. For CLD difference and AC difference (%), mean values within columns with different letters are significantly different at $p < 0.01$

CLD chain length distribution, *SCP* short chain proportion (DP 6–10), *ICP* intermediate chain proportion (DP 11–24), *AC* amylose content

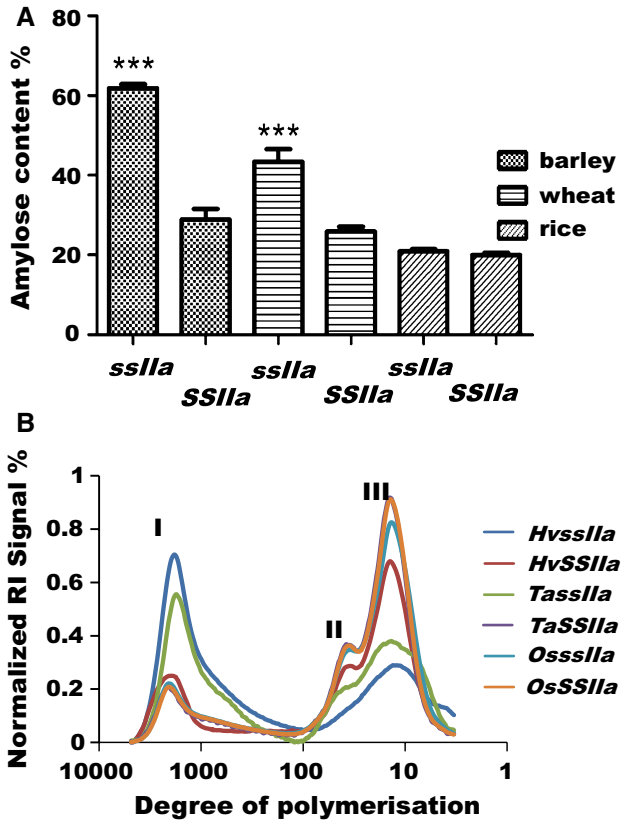

Fig. 6 Comparison analyses of amylose contents of *ssIIa* mutants or inactive SSIIa variant and *SSIIa* wild types of three cereals by size-exclusion chromatography (SEC). **a** Five lines for each genotype of three cereals were analysed. Assays were repeated twice for each sample. The *error bars* indicate SEM. The identities of *bars* are indicated beneath the figure, and three different patterns for three cereals are shown on the *right* side of the figure. *Columns* with *** show significant difference at $p < 0.001$. **b** Distribution of normalised refractometer index (RI) signals of debranched starches from three cereals. Peak I is amylose peak; peak II is long-chain amylopectin peak; and peak III is amylopectin peak. The identity of *lines* is indicated on the *right* side of the figure

role of SSIIa was more critical inside the starch granules than in the soluble phase.

In the present study, the expression levels of mRNAs for *SSI, SBEIIa* and *SBEIIb* in mutants and inactive SSIIa variant remained comparable to that of the wild type, suggesting if the translational efficiency was not altered, that the amount of the corresponding proteins in *ssIIa* mutants or inactive SSIIa variant would be comparable with that of the wild-type grains. At the protein level in the soluble fraction of the endosperm, the abundance of SSI and SBEIIb proteins significantly increased in *ssIIa* mutants and inactive SSIIa variant; however, the abundance of SSI and SBEIIb proteins inside starch granules in mutants and inactive SSIIa variant was sharply reduced compared to the wild types. The amount of SBEIIa proteins was comparable in the soluble fraction between the *ssIIa* mutants or inactive SSIIa variant and *SSIIa* wild types, but its concentration inside the starch granules was sharply reduced in barley and wheat *ssIIa* mutants and rice had very low levels of SBEIIa even in the wild type. In barley and wheat, the majority of SSI, SBEIIa and SBEIIb protein that normally associates with starch failed to enter the starch granule and remained in the soluble phase of amyloplast, and in rice these changes were smaller in magnitude.

The amount of SSI and SBEIIb remaining bound to the starch granule in the mutants and inactive SSIIa variant can mostly be explained by the level and activity of SSIIa in each cereal since it has been proposed that SSIIa may act as the central protein in the formation of a trimeric SSI-SSIIa-SBEIIb protein complex in maize (Liu et al. 2012b). In barley and wheat mutants no functional full-length SSIIa protein is produced due to a stop codon and indel mutations, respectively. However, in *japonica* rice the SSIIa protein variant is present only at reduced levels compared to *indica* rice and the starch-binding affinity of the protein is also reduced (Umemoto and Aoki 2005). In the absence

of SSIIa, the mechanism of how some (25 % of wild-type levels) SBEIIb is found inside the starch granule in *TassIIa* mutants remains to be further studied. Since SBEIIa is a minor branching enzyme inside starch granules compared with SBEIIb, most of the protein possibly exists in the soluble fraction of wheat (Regina et al. 2005). Hence, the small increase in abundance could not be measured and analysed statistically for the SBEIIa proteins in the soluble fraction.

Comparison of starch properties among different cereals with defects in SSIIa

The increase of amylose content, the alterations in the amylopectin fine structure and the reduction of amylopectin content in different cereal *ssIIa* mutants and inactive SSIIa variant has been reported before (Konik-Rose et al. 2007; Kramer et al. 1958; Morell et al. 2003; Umemoto and Aoki 2005; Zhang et al. 2004). In the current study the changes in amylose content have been further defined by quantitating the long linear amylose chains of debranched starches by SEC. These results were similar to that determined by iodine binding and HPLC of native starches in published papers with barley having the highest amylose, wheat intermediate and rice the lowest levels (Konik-Rose et al. 2007; Morell et al. 2003; Umemoto et al. 2004). The amylose content was negatively correlated with the amount of SSI and SBEIIb remaining inside the starch granules in the *ssIIa* mutants and inactive SSIIa variant. That is, mutants and inactive SSIIa variant containing low SSI and SBEIIb content inside the starch granules produce starch with less intermediate amylopectin chains, low amylopectin content and high amylose content.

Although starch biosynthetic enzymes are supposed to be non-functional after their entrapment inside the amylopectin molecules (Denyer et al. 1993), the abundance and distribution of them between the granule and stroma could still reflect the impact and involvement of each enzyme during the starch accumulation process. It has been suggested that SSI-SSIIa and SBEII trimeric protein complex is involved in the synthesis of amylopectin at the surface of amylopectin molecules and is then trapped inside of starch granules (Liu et al. 2012b). Losing any of SSI, SSIIa and SBEIIb can change distribution patterns of the short and intermediate length chains of amylopectin of cereal grains (Fujita et al. 2006; Nishi et al. 2001; Morell et al. 2003; Umemoto et al. 2004; Yamamori et al. 2000). These studies on SSIIa mutants showed that SSIIa elongates the short chains which are synthesised by SSI. When SSIIa is defective, most of the short chains synthesised by SSI can hardly be elongated, showing the percentage of short chains remarkably increases. SSI in soluble fraction may be also active in amylopectin biosynthesis on the surface of amylopectin molecules to synthesise more short chains in amylopectin. Although SSIIa is believed to directly involved in the elongation of short amylopectin length chains to the intermediate length (Cao et al. 2000; Nakamura et al. 2005b), the remarkable abnormal starch structure produced by eliminating SSIIa is most likely the result of a combination of the absence of SSI, SSIIa, SBEIIa and SBEIIb inside starch granules rather than only the lack of SSIIa activity per se. To further prove this, the functional interactions of SSI, SBEIIa and SBEIIb need to be characterised using double or triple protein deficient mutants.

Proposed mechanisms for the effects of ssIIa mutations on starch biosynthesis in the endosperms of cereals

Through analysis of *ssIIa* mutants and inactive SSIIa variant for barley, wheat, rice and maize (this work and Liu et al. 2012b), certain mechanisms are proposed here for *ssIIa* mutations and inactive SSIIa variant on starch biosynthesis in the endosperms of different cereals (Fig. 7). As the presence of SBEIIa protein inside the starch granules is different among four cereals (i.e. at low abundance for barley and wheat, and absent for rice and maize), SBEIIa is not presented in the current model. In wild-type cereals, SSIIa can form protein complexes with SSI and SBEIIb effectively to synthesise wild-type amylopectin and maintain the ratio between amylose and amylopectin (Fig. 7a). In *OsssIIa* inactive SSIIa variant endosperm, inefficient binding of ssIIa proteins with starch due to an amino acid change in the protein of the inactive SSIIa variant also causes protein complexes to bind inefficiently to starch (Umemoto et al. 2004) (Fig. 7b), producing relatively less amylopectin leading to a slight proportional increase in amylose content. In the maize *ssIIa* sugary mutant endosperm, although ssIIa proteins completely lose affinity with starch due to a mutation in the starch-binding domain, the proteins can still assemble into complexes in the amyloplast stroma (Fig. 7c). However, this leads to the inability of the other components of the protein complex to become starch granule-bound, therefore, leads to a reduction in functional branching enzyme activity within the complex and increase in relatively higher amylose starch (Liu et al. 2012b). In *HvssIIa* and *TassIIa* mutants, no SSIIa protein is produced due to stop or indel mutations; thus the absence of any trimeric complex prevents the binding of SSI and SBEIIb proteins to the starch granules (Fig. 7d). In this last scenario, amylopectin synthesis is significantly interrupted, therefore shifting the starch biosynthetic pathway into producing more amylose.

As shown in Fig. 7, SSIIa plays similar function in the synthesis of amylopectin in cereal endosperm, which demonstrates that SSIIa in different cereals may have a comment evolutionary path. It was reported that all five starch

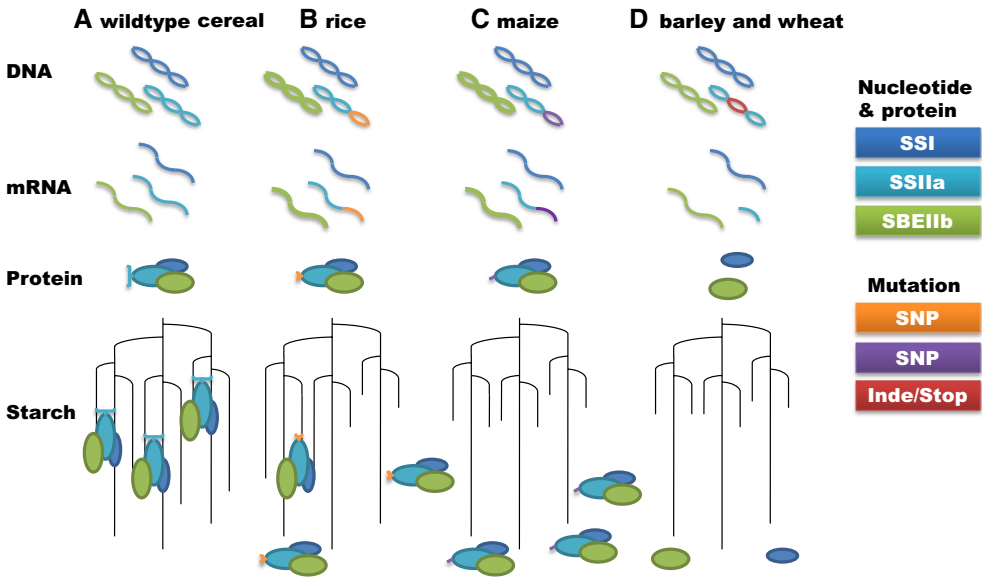

Fig. 7 Mechanism of *ssIIa* mutations or inactive SSIIa variant in the endosperms of cereals. *A* wild-type cereals, *B OsssIIa*, *C ZmssIIa*, *D HvssIIa* & *TassIIa*. Molecular levels are indicated on the *left*. Identities of different *colours* are labelled on the right.} and / indicate the different affinity of SSIIa with starch

synthase subfamilies contain genes from both seed plants and mosses and/or lycophytes, suggesting that the main characteristic of this family in land plants was established before the origin of seed plants (Yang et al. 2013). Within seed plants, SSII subfamilies in monocots have three isoforms (SSIIa, SSIIb and SSIIc) through gene duplication, but only isoform of SSII in eudicots is reported (Hirose and Terao 2004; Cheng et al. 2012), suggesting that the gene duplication of SSII in monocots after diversification between monocots and eudicots. SSIIa maily expresses in cereal endosperm, which is further diversified from SSIIc in monocots and SSII in eudicots.

Conclusion

This study demonstrated that the distribution of SSI, SSIIa, SBEIIa and SBEIIb inside the starch granules in SSIIa mutants and inactive SSIIa variant of barley, wheat and rice is the result of post-translational regulation, rather than regulations at transcriptional or translational levels. The abundance of these four proteins inside the starch granules was correlated with the abundance of SSIIa inside the starch granules. The majority of SSI, SBEIIa and SBEIIb that failed to bind to the starch granules were found remaining in the soluble stroma phase due to the complete absence (barley and wheat) or low abundance (rice) of SSIIa proteins in starch granules. This indicates that SSIIa possibly functions as a post-translational regulator in starch biosynthesis in the endosperm of cereals through the distribution of SSI, SSIIa, SBEIIa and SBEIIb inside the starch granules.

Author contribution statement JL carried out the genotyping, analysis of mRNA expression, SDS-AGE, immunoblot, starch CLD, AC analysis, interpretation of data and drafting the manuscript. BK made contribution to the immunodetection of starch synthetic enzymes. OL analysed CE and SEC data. VMB contributed to SEC analysis. GJT and MLC contributed to Mass spectrometry analysis. NMU contributed to producing rice RIL lines. SAJ made substantial contributions to conception, design and revising the manuscript critically for important intellectual content. TJT, MJE, AM and MKM made substantial contributions to conception and design. ZL made substantial contributions to conception and design, analysis and interpretation of data, revising manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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