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RESEARCH PAPER

Elongated phytoglycogen chain length in transgenic rice endosperm expressing active starch synthase IIa affects the altered solubility and crystallinity of the storage α-glucan

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Abstract

The relationship between the solubility, crystallinity, and length of the unit chains of plant storage α-glucan was investigated by manipulating the chain length of α-glucans accumulated in a rice mutant. Transgenic lines were produced by introducing a cDNA for starch synthase IIa (SSIIa) from an *indica* cultivar (*SSIIa*^{I}, coding for active SSIIa) into an isoamylase1 (ISA1)-deficient mutant (*isa1*) that was derived from a *japonica* cultivar (bearing inactive SSIIa proteins). The water-soluble fraction accounted for >95% of the total α-glucan in the *isa1* mutant, whereas it was only 35–70% in the transgenic *SSIIa*^{I}*/isa1* lines. Thus, the α-glucans from the *SSIIa*^{I}*/isa1* lines were fractionated into soluble and insoluble fractions prior to the following characterizations. X-ray diffraction analysis revealed a weak B-type crystallinity for the α-glucans of the insoluble fraction, while no crystallinity was confirmed for α-glucans in *isa1*. Concerning the degree of polymerization (DP) ≤30, the chain lengths of these α-glucans differed significantly in the order of *SSIIa*^{I}*/isa1* insoluble > *SSIIa*^{I}*/isa1* soluble > α-glucans in *isa1*. The amount of long chains with DP ≥33 was higher in the insoluble fraction α-glucans than in the other two α-glucans. No difference was observed in the chain length distributions of the β-amylase limit dextrins among these α-glucans. These results suggest that in the *SSIIa*^{I}*/isa1* transgenic lines, the unit chains of α-glucans were elongated by SSIIa^{I}, whereas the expression of SSIIa^{I} did not affect the branch positions. Thus, the observed insolubility and crystallinity of the insoluble fraction can be attributed to the elongated length of the outer chains due to SSIIa^{I}.

Key words: Amylopectin, endosperm, isoamylase, phytoglycogen, starch synthase, transgenic rice.

Introduction

Starch biosynthesis is catalysed by four known classes of enzymes: ADP-glucose pyrophosphorylases (AGPases), starch synthases (SSs), starch branching enzymes (BEs), and starch debranching enzymes (DBEs) (Smith *et al*., 1997; Myers *et al*., 2000; Nakamura, 2002; Ball and Morell, 2003). SS elongates the α-1,4 glucosidic chains of amylopectin and contains the greatest number of isozymes found in green plants. Each SS class plays a distinct role in starch biosynthesis and exhibits

Abbreviations: β-LD, β-amylase limit dextrin; DP, degree of polymerization; DSC, differential scanning calorimeter; ISA, isoamylase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; SEM, scanning electron microscopy.

tissue and substrate specificities. Several SS genes exist in green plants. Among these, the functions of granule-bound starch synthase I (GBSSI), SSI, SSIIa, and SSIII(a) are relatively well known. GBSSI is involved in elongating amylose and extra long chains (ELCs) of amylopectin (Sano, 1984; Takeda *et al*., 1987; Hanashiro *et al*., 2008). SSI generates DP (degree of polymerization) 8–12 chains from DP 6–7 chains that emerge from the branch point in the A and B_1 chain of amylopectin (Fujita *et al*., 2006, 2008). SSIII(a) functions to elongate the long $B_{2–3}$ chains that connect multiple clusters of amylopectin (Inouchi *et al*., 1983; Fulton *et al*., 2002; Ral *et al*., 2006; Fujita *et al*., 2007; Borén *et al*., 2008).

SSII studies have been performed in maize (*sugary2*: Takeda and Preiss, 1993; Zhang *et al*., 2004), wheat (SGP-1 null: Yamamori *et al*., 2000), rice (*japonica* varieties: Umemoto *et al*., 2002; Nakamura *et al*., 2005*a*); barley (*sex6*: Morell *et al*., 2003), sweet potato (cv. Quick sweet: Katayama *et al*., 2002; Kitahara *et al*., 2005), and *Arabidopsis* (*Atss2*: Zhang *et al*., 2008). These SSII-deficient mutants also accumulated a modified amylopectin, which was enriched with short chains with DP ≤12, instead of intermediate length chains with DP 13–24 (mostly B_1 chains). The proportion of longer B chains (DP ≥24; B_2 and B_3) was unchanged in these plant species, except for *rug5* in pea embryos (Craig *et al*., 1998). These findings suggest that SSII(a) performs a uniform function in these plant species. It elongates short chains with DP ≤12 to intermediate length chains within amylopectin clusters. This has a tremendous impact on the gelatinization temperature of starch (Craig *et al*., 1998; Edwards *et al*., 1999; Lloyd *et al*., 1999; Yamamori *et al*., 2000; Umemoto *et al*., 2002; Nakamura *et al*., 2002, 2005*a*; Zhang *et al*., 2004). Concerning rice, most *indica* rice varieties possess active SSIIa. In contrast, most *japonica* rice cultivars have markedly low or no SSIIa activity caused by three SNPs (single nucleotide polymorphisms) in the *SSIIa* gene (Nakamura *et al*., 2002, 2005*a*).

Recent analyses of ISA1-deficient mutants (*sugary1* or *isa1* mutants) indicate that the fourth class of enzymes related to starch biosynthesis, designated DBEs, also play an essential role in starch biosynthesis. These mutants accumulate highly branched soluble α-glucan (phytoglycogen) instead of amylopectin in maize (Pan and Nelson, 1984; James *et al*., 1995), rice (Nakamura *et al*., 1996, 1997), barley (Burton *et al*., 2002), and *Chlamydomonas* (Mouille *et al*., 1996). James *et al*. (1995) identified the maize *Sugary1* (*Su1*) gene by transposon tagging of *su1* mutants. This gene encodes an ISA-type DBE.

Some allelic *isa1* mutant rice lines have been reported and they exhibit different phenotypes of accumulated glucans, either mild or severe phenotypes. The mild phenotype *isa1* lines accumulate specific amylopectin that contains an abundance of short chains (*sugary*-amylopectin) and amylose within the outer region of the endosperm. In contrast, the phytoglycogen is located in the inner region of the endosperm. The severe phenotype *isa1* lines accumulate primarily phytoglycogen instead of starch in the whole endosperms (Nakamura *et al*., 1997; Kubo *et al*., 1999). The severe *isa1* mutants have not been identified in cereal crops other than rice, and an exceptional example has been reported for transient starch synthesis in *Arabidopsis* leaves (Zeeman *et al*., 1998). Wong *et al*. (2003) analysed the structure and physicochemical properties of the α-glucans that accumulate in the rice allelic *isa1* lines that exhibit different severities of the *sugary1* phenotype. Phytoglycogen was recovered from supernatants using low-speed centrifugation, indicating that phytoglycogen is a soluble α-glucan. Phytoglycogen consists of significantly more short chains of DP ≤10 and fewer chains of 11 ≤DP ≤24 when compared with wild-type amylopectin. In phytoglycogen, the quantity of long chains with DP ≥37 corresponding to $B_{2–3}$ chains of amylopectin is significantly decreased when compared with amylopectin. Phytoglycogen is composed of multiple components with smaller molecular weights than amylopectin. A greater proportion of short chains compared with normal amylopectin in the *isa1* allelic lines contributes to defective A-type crystallinity, and a lower gelatinization temperature and decreased enthalpy when analysed by X-ray diffractometry and differential scanning calorimetry (DSC), respectively (Wong *et al*., 2003).

Green plants have evolved the capacity to synthesize highly organized branched α-glucans as amylopectin with tandem cluster structure, whereas animals and bacteria continue to produce random branched glycogen. Throughout the long evolution of plants, a wide variety of α-glucan structures can be distinguished. These range from the primitive cyanobacterial glycogen to the highly organized amylopectin typical of green plants. Intermediate α-glucan structures, such as cyanobacteria-starch and semi-amylopectin, have been identified in unique cyanobacteria including *Cyanobacterium* sp MBIC10216 and in some species of Rhodophyta such as *Porphyridium purpureum* (Nakamura *et al*., 2005*b*; Deschamps *et al*., 2008; Shimonaga *et al*., 2008). Amylopectin and glycogen are both composed of branched α-glucans that contain α-1,4 and α-1,6 glucosidic linkages. However, the solubility and crystallinity of these α-glucans are quite different. The solubility of α-glucans greatly affects the osmotic pressure of the cell and the α-glucan storage mechanism in each organism.

Starches are roughly divided into two types of crystallinity. Cereal endosperm starch displays the A-type X-ray diffraction pattern, and potato starch displays the B-type diffraction pattern. Starches containing amylopectin of relatively short average branch chain lengths (DP 23–29) display the A-type X-ray pattern, while other starches containing amylopectin of relatively longer branch chains (DP 30–44) displays the B-type X-ray pattern (Hizukuri, 1985). Studies concerning the relationship between crystallinity and starch structure have suggested that (i) a chain length of at least DP ≥10 is necessary for the formation of parallel glucan double helices and crystallinity (Gidley and Bulpin, 1987); (ii) a relatively large proportion of short chains with DP ≤9 decreases the crystallinity of starch (Fujita *et al*., 2003; Wong *et al*., 2003); and (iii) crystallinity disappears when starch is gelatinized. The specific structural characteristics, namely chain length, branch points, and molecular weight, that are necessary for the insolubility and crystallinity of α-glucans remain to be resolved. However, the tandem cluster structure of amylopectin is recognized as being very important.

To investigate the relationship between the solubility, crystallinity, and length of unit chains of plant storage α-glucans, this study generated transgenic rice (*SSIIa*^{I}*/isa1*) exhibiting elongated outer chains of phytoglycogen. These plants were produced by introducing the active *SSIIa* gene of *indica* rice (*SSIIa*^{I})

into the *japonica* rice *sugary-1* mutant (*isa1*) which contains inactive SSIIa. Analyses of the structure and physicochemical properties of these α-glucans permitted the comparison of a line lacking both SSIIa and ISA1 with a line lacking only ISA1. The requirements for the insolubility and crystallinity of α-glucans are discussed.

Materials and methods

Plant materials

The rice cultivars Nipponbare and Taichung 65 (T65) (*japonica* cultivars) and IR36 (*indica* cultivars) were included as wild-type plants in this study. A severe type of the *sugary-1* (*sug-1*) mutant (isoamylase1-deficient mutant, *isa1*) line, *EM914* (Nakamura *et al*., 1997), was used as the host mutant. *EM914* is a product of *N*-methyl-*N*-nitrosourea (MNU) mutagenesis of the rice cultivar T65 (Satoh and Omura, 1979). These rice lines were grown during the summer months in a paddy field and greenhouse at Akita Prefectural University.

Generation of transgenic rice lines

A DNA construct containing the *SSIIa* cDNA from *indica* cultivar IR36 (Nakamura *et al*., 2005*a*) under the control of the rice Wx^a promoter (Supplementary Fig. S1 available at JXB online; Utsumi *et al*., 2011) was introduced into *EM914* (*isa1*) by *Agrobacterium tumefaciens* EHA105-mediated transformation (Hood *et al*., 1993). Procedures for rice tissue culture, transformation, and selection were as described previously (Kubo *et al*., 2005). A total of seven individual T_0 progeny lines were isolated from the transformation. Three T_1 seeds were randomly chosen from each T_0 plant. These seeds were independently analysed for endosperm amylopectin chain length distribution using capillary electrophoresis as described below. Five randomly chosen T_1 seeds of four T_0 transformed lines were grown, and their seeds (T_2) were examined for amylopectin chain length distribution. Western blotting was also conducted using SSIIa antiserum (Nakamura *et al*., 2005*a*). Four homozygous lines (*SSIIa^l/isa1-#1*, *#2*, *#7*, and *#20*) were selected and their seeds (T_3) were used for further studies.

Transgenic rice lines were grown during the summer months in a greenhouse at Akita Prefectural University.

Native-PAGE/activity staining and immunoblotting

Native-PAGE/activity staining of DBE and BE was performed using the methods of Fujita *et al*. (1999) and Yamanouchi and Nakamura (1992), respectively. SS activity staining was performed on 7.5% (w/v) acrylamide slab gels containing 0.8% (w/v) oyster glycogen (G8751, Sigma) according to Nishi *et al*. (2001) with the modification that 0.5 M citrate was included in the reaction mixture.

Preparation of soluble protein, loosely bound protein, and tightly bound protein from the mature endosperm was performed according to the methods of Fujita *et al*. (2006). Immunoblotting was performed according to the methods of Fujita *et al*. (1999) using antiserum raised against the peptide fragment APKPKATRSSPIPA of SSIIa in rice cultivars. This peptide sequence is common in *indica* (Kasalath and IR36) and *japonica* (Nipponbare and Kinmaze) cultivars (Nakamura *et al*., 2005*a*).

Preparation of stereomicrographs of kernel cross-sections

Dehulled rice seeds, whose embryos were removed at the mature stage, were soaked in distilled water for 16 h and cut across the short axis with a razor blade. The cross-sections were stained with 0.5% KI/0.05% I_2 solution and observed under a stereo microscope (Olympus SZX7, Tokyo, Japan).

Analysis of the starch granules of endosperm

An estimation of the amount of soluble and insoluble α-glucans from rice endosperm was performed as described in Tanaka *et al*. (2004) and Fujita *et al*. (2003), respectively. Total α-glucans from a transgenic rice line (*SSIIa^l/isa1-#20*) and parent mutant (*EM914*) were prepared by grinding dehulled, seeds, whose embryos had been removed, at the mature stage (~0.5 g) with a mortar and a pestle. Soluble and insoluble fractions were prepared from the total α-glucan as follows. A suspension of total α-glucan in 5 ml of distilled water was subjected to low-speed (600 *g*) centrifugation at 20 °C for 10 min. The precipitate was washed twice with 5 ml of distilled water and the resulting precipitate (i.e. the insoluble fraction) was designated *SSIIa^l/isa1-#20* insoluble fraction (#20 Insoluble). The first 600 *g* supernatant was combined with those from the following washes and these soluble fractions were designated *SSIIa^l/isa1-#20* soluble fraction (#20 Soluble). The insoluble and soluble fractions of *#20* were dried under reduced pressure. Measurements of the thermal properties of endosperm starch by differential scanning calorimetry (DSC; DSC-6100, Seiko instrument) and X-ray diffraction were performed as described previously (Fujita *et al*., 2003, 2006).

Starch granules for scanning electron microscopy (SEM) observation were purified using Percoll (Amersham Biosciences) according to the method of Shimonaga *et al*. (2008). Two (for T65) to five (for *EM914* and *#20* transgenic rice lines) dehulled rice seeds, whose embryos had been removed, were ground with a mortar and pestle. The ground tissue was suspended in 0.5 ml of distilled water, layered onto 1.5 ml of Percoll, and centrifuged at 30 000 *g* for 20 min at 4 °C. The starch pellet was washed with 100% ethanol and dried under pressure. Purified starch granules were coated with gold using a fine coater (JEOL JFC-1200) for 120 s. The morphology of the starch granules was examined by SEM (JEOL-500, Tokyo, Japan). SEM was performed in a secondary electron mode at 15 kV. For endosperm observations, dried rice seeds were cut across the short axis with a razor blade. The surface was sputter-coated with gold and observed using SEM with the same conditions described above.

Analyses of α-glucan structure

Phytoglycogen and α-glucan specimens for chain length distribution analyses by high-performance size-exclusion chromatography (HPSEC) were prepared as follows: rice seeds (0.3–1.0 g) were ground with a mortar and pestle and the powder was suspended in 3–4 ml of distilled water. The suspension was centrifuged at 600 *g* and 20 °C for 10 min. Three volumes of methanol were added to the supernatant, and the mixture was kept at 4 ºC overnight. The precipitate was collected by centrifugation at 3 000 *g* and 4 °C for 10 min. The precipitate was washed by suspension in 2 ml of ice-cold methanol and centrifugation at 10 000 *g* and 4 °C for 10 min. Sample drying was conducted in a centrifugal vacuum evaporator (designated #20 soluble fraction). For #20, the precipitate that was collected by centrifuging at 600 *g* was further purified by dissolving in 6 ml of 100% dimethylsulphoxide at 37 °C overnight. The sample was then centrifuged twice at 600 *g* and ambient temperature for 10 min. The supernatant was precipitated with 3 vols of methanol as described above and the dried sample was designated #20 insoluble fraction.

The chain length distributions of α-glucans from endosperm were analysed using the fluorescence capillary electrophoresis (FCEP) method of O'Shea and Morell (1996) and Fujita *et al*. (2001) in a P/ACE MDQ Carbohydrate System (Beckman Coulters, CA, USA). The distributions were also analysed by HPSEC of debranched α-glucans labelled with 2-aminopyridine as previously described (Fujita *et al*., 2009). The preparation and debranching of β-amylase limit dextrin (β-LD) of the α-glucans was conducted according to Hanashiro *et al*. (2011), and the chain length distribution was analysed with HPSEC in the same manner as was used for the native α-glucans.

Molecular size separation of starch from wild-type, soluble, and insoluble fractions from the transgenic rice line *#20* and total α-glucans from *EM914* by Sephacryl S-1000SF chromatography was performed according to the method of Kubo *et al*. (1999). After chromatography, an aliquot of each fraction was used to measure the carbohydrate content

by an enzymatic method (Nakamura and Miyachi, 1982) and to measure the λ_{max} value of the glucan–iodine complex (Fujita *et al*., 2007). Commercial pullulan standards with defined average molecular weights (Shodex, Showa denko) were used to calibrate the column.

Results

Generation of the SSIIa^{I}/isa1 *transgenic rice lines*

The *SSIIa* cDNA derived from the *indica* cultivar IR36 (*SSIIa*^{I}; Nakamura *et al*., 2005*a*) was introduced into the severe *isa1* mutant, *EM914* (Nakamura *et al*., 1997), whose endogenous SSIIa is inactive (Table 4), using the *Agrobacterium* method. The isozyme activity related to starch biosynthesis in the developing endosperm (~15 d after flowering) of the transgenic rice lines (*SSIIa*^{I}*/isa1*) was analysed by native-PAGE/DBE, BE, and SS activity staining (Fig. 1). ISA1 activity was significantly reduced, and a pleiotropic effect of reduced pullulanase (PUL) activity, which is the other type of debranching enzyme, was observed in four *SSIIa*^{I}*/isa1* lines and the host *EM914* (Fig. 1; Kubo *et al*., 1999). The activity of three BE isozymes (BEI, BEIIa, and BEIIb), Pho1, and two SS isozymes (SSI and SSIIIa) was also reduced in these lines compared with wild-type plants (Fig. 1). This result was consistent with the well-known phenotype of the *isa1* background, which typically displays significantly reduced starch biosynthesis.

The SSIIa activity band was difficult to detect using native-PAGE/SS activity staining. Therefore, immunoblotting of three different fractions (soluble protein, loosely bound protein, and tightly bound protein) prepared from T_3 dry seeds of the *SSIIa*^{I}*/isa1* lines was conducted using an antiserum raised against SSIIa. This immunoblot confirmed the expression of the introduced *SSIIa*^{I} gene (Fig. 2). SSIIa^{I}, derived from *indica* rice, was previously detected in the tightly bound protein fraction. Inactive SSIIa, derived from *japonica* rice, was detected in the soluble protein and/or loosely bound protein fractions (Fig. 2; Nakamura *et al*., 2005*a*). Faint SSIIa bands in individual T_3 seeds in *#1* and *#7* were detected exclusively in the soluble protein and loosely bound protein fractions. Strong SSIIa bands in *#2* (1, 2, and 4) and *#20* (2, 3, 4, 5, and 6) were detected in the tightly bound protein fraction, as well as in the soluble protein and loosely

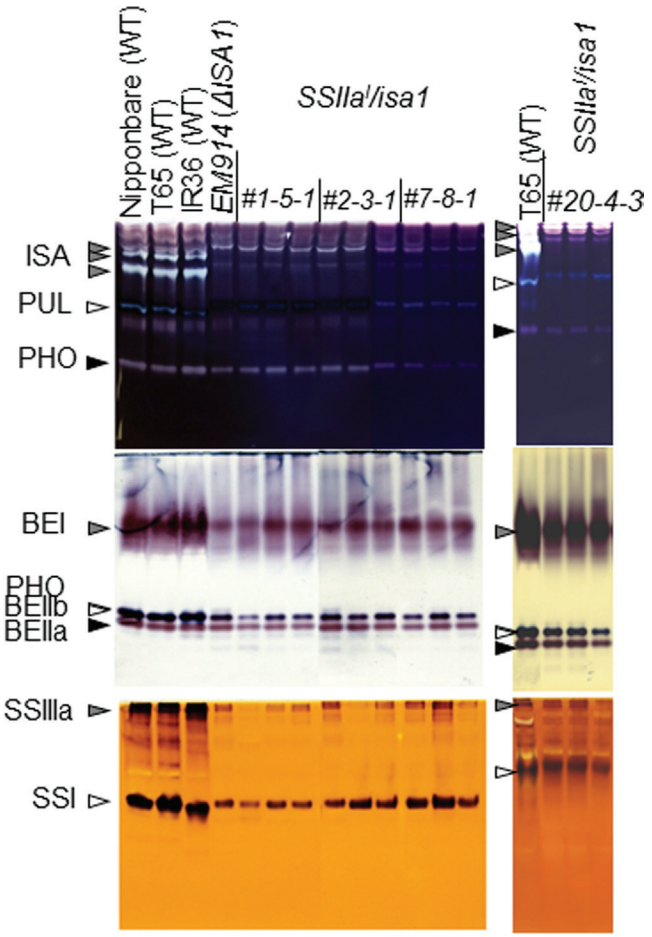

Fig. 1. Native-PAGE/activity staining of debranching enzyme (DBE), branching enzyme (BE), and starch synthase (SS) in developing endosperm of transgenic and wild-type rice. The ISA (isoamylase), PUL (pullulanase), PHO (phosphorylase), BEI, BEIIa, BEIIb, SSIIIa, and SSI activity bands are indicated by arrowheads. Crude extracts were prepared by adding 3 vols of grinding solution per fresh weight of the developing endosperm. The volume of crude extract applied to the native gels in DBE, BE, and SS were 5, 2, and 8 µl, respectively.

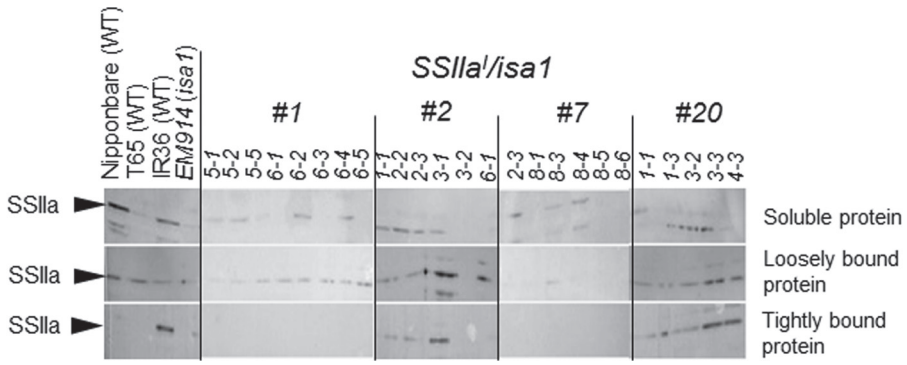

Fig. 2. Immunoblotting of three protein fractions, soluble protein, loosely bound protein, and tightly bound protein, prepared from developing endosperm in transgenic rice lines (*SSIIa*^{I}*/isa1*) using antiserum raised against a peptide of *Os*SSIIa (Nakamura *et al*., 2005*a*). The numbers in the lines show the individual T_3 seeds.

bound protein fractions (Fig. 2). These results suggest that the introduced *SSIIa*^{I} gene was highly expressed in the *#2* and *#20* lines. T_3 seeds in the *#2* and *#20* lines and in the *#1* and *#7* lines were used for further studies to represent transgenic rice lines expressing the gene at high and low levels, respectively.

Characterization of the SSIIa^{I}/isa1 *transgenic rice lines*

Cross-sections of the mature kernels of *SSIIa*^{I}*/isa1* and the parent lines exhibited blue to purple coloration with iodine solution (Fig. 3). Nearly all of the endosperm cells of *isa1* (*EM914*) did not exhibit such coloration with iodine (Fig. 3; Nakamura *et al*., 1997). In contrast, endosperm cells of the wild type (T65) were completely stained. This result indicates that the *isa1* line accumulates phytoglycogen that contains plenty of short chains in the whole endosperm cells rather than accumulating starch. In contrast, the outer layers of the *SSIIa*^{I}*/isa1* endosperm tissue exhibited blue to purple coloration, whereas the inner region of the kernel did not in the *#2* and *#20* lines, which had high expression levels of the *SSIIa*^{I} gene (Fig. 2). On the other hand, very little of the endosperm exhibited purple coloration with iodine in *#1* and *#7*, lines which had low *SSIIa*^{I} expression levels.

The weight of the dehulled grain from the *isa1* line was approximately half (*EM914*, 58.2%) of the wild-type weight (Table 1). The weights of the *SSIIa*^{I}*/isa1* lines were reduced in comparison with the parent mutant (37.6–54.6% of the wild type), and the values were not related to the level of *SSIIa*^{I} gene expression.

α-Glucan samples were separated into soluble and insoluble fractions by centrifugation at 600 *g* for 10 min at 20 °C (see the Materials and methods). The amount of insoluble α-glucan in the wild type (T65) was 97.4% of the total amount of α-glucan contained in the endosperm. In contrast, the amount of insoluble α-glucan in *isa1* (*EM914*) was only 3.1% (Table 1; Wong *et al*., 2003). The amount of insoluble α-glucan in the *SSIIa*^{I}*/isa1* lines increased in comparison with the parent mutant (26.6–65.4% of the wild type, Table 1). The *#2* and *#20* lines, which exhibited high *SSIIa*^{I} gene expression, contained markedly high quantities of insoluble α-glucans (51.0–65.4%). Lines *#1* and *#7*, which exhibited low *SSIIa*^{I} gene expression, had relatively low quantities of insoluble α-glucans (26.6–34.5%).

The fine structure of α-glucans

To evaluate the effect of *SSIIa*^{I} gene expression on the fine structure of phytoglycogen, the chain length distribution of the isoamylolysates of the total α-glucans (see the Materials and methods) in the endosperm was analysed by the FCEP method using the *SSIIa*^{I}*/isa1* and parent mutant lines (Fig. 4). The shorter chains with DP 3–10 and the longer chains with DP ≥11 were significantly increased and decreased, respectively, in the *isa1* line when compared with the wild types (Fig. 4A, B; Nakamura *et al*., 1997; Wong *et al*., 2003). The shorter chains with DP ≤10 and the intermediate chains with 11 ≤DP ≤30 were decreased and increased, respectively, in the four lines of *SSIIa*^{I}*/isa1* when compared with *isa1* (Fig. 4B). Of the *SSIIa*^{I}*/isa1* lines, *#2* and *#20* exhibited high expression of *SSIIa*^{I} and high quantities of insoluble α-glucans. For these lines, the extent of these changes in chain length distribution pattern was significantly large. It is worth noting that the long chains with DP ≥33 connecting the amylopectin clusters

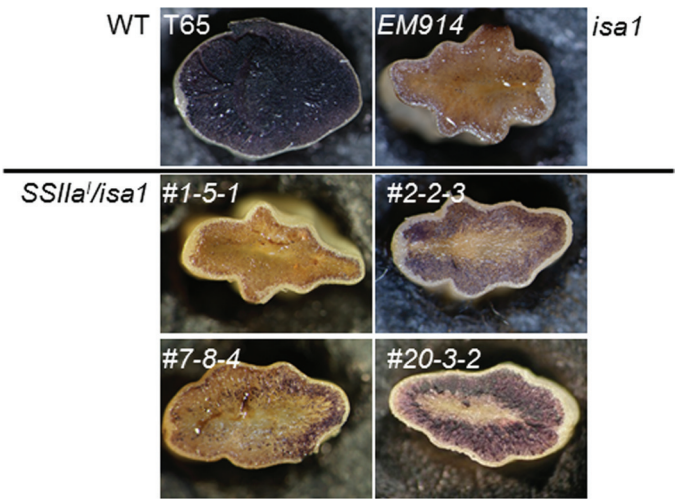

Fig. 3. Stereomicrographs of iodine-stained cross-sections of water-absorbed mature seeds of transgenic lines, *SSIIa*^{I}*/isa1 (#1*, *#2*, *#7*, and *#20*), the host *isa1* mutant (*EM914*), and the wild type (T65 and IR36).

Table 1. *Dehulled grain weight, and soluble and insoluble α-glucan content in dry seeds of transgenic rice and their parents*

Lines		Dehulled grain weight[a] (mg)		Soluble α-glucan[b] (mg)	Insoluble α-glucan[b] (mg)	% of insoluble fraction of total α-glucan
Taichung 65	Wild type	19.4 ± 1.5	(100)[c]	0.38 ± 0.15	14.2 ± 0.73	97.4
EM914	*isa1*	11.3 ± 0.2	(58.2)	3.80 ± 0.89	0.12 ± 0.06	3.1
#1	*SSIIa*^{I}*/isa1*	8.1 ± 1.0	(41.8)	2.87 ± 0.38	1.04 ± 0.12	26.6
#2 (3)[d]	*SSIIa*^{I}*/isa1*	10.6 ± 0.2	(54.6)	3.49 ± 0.43	3.63 ± 0.53	51.0
#2 (4)	*SSIIa*^{I}*/isa1*	8.9 ± 1.0	(45.9)	1.65 ± 0.39	2.54 ± 0.23	60.6
#7	*SSIIa*^{I}*/isa1*	7.8 ± 1.3	(40.2)	3.26 ± 0.46	1.72 ± 0.16	34.5
#20 (4)	*SSIIa*^{I}*/isa1*	7.3 ± 0.4	(37.6)	1.58 ± 0.53	2.05 ± 0.33	56.5
#20 (6)	*SSIIa*^{I}*/isa1*	9.3 ± 1.0	(47.9)	1.97 ± 0.30	3.72 ± 0.19	65.4

[a] Mean of 20 seeds.
[b] *n*=3.
[c] Percentage of the wild type.
[d] These numbers correspond to the sample number from Fig. 2, which originated from the same T_3 seeds.

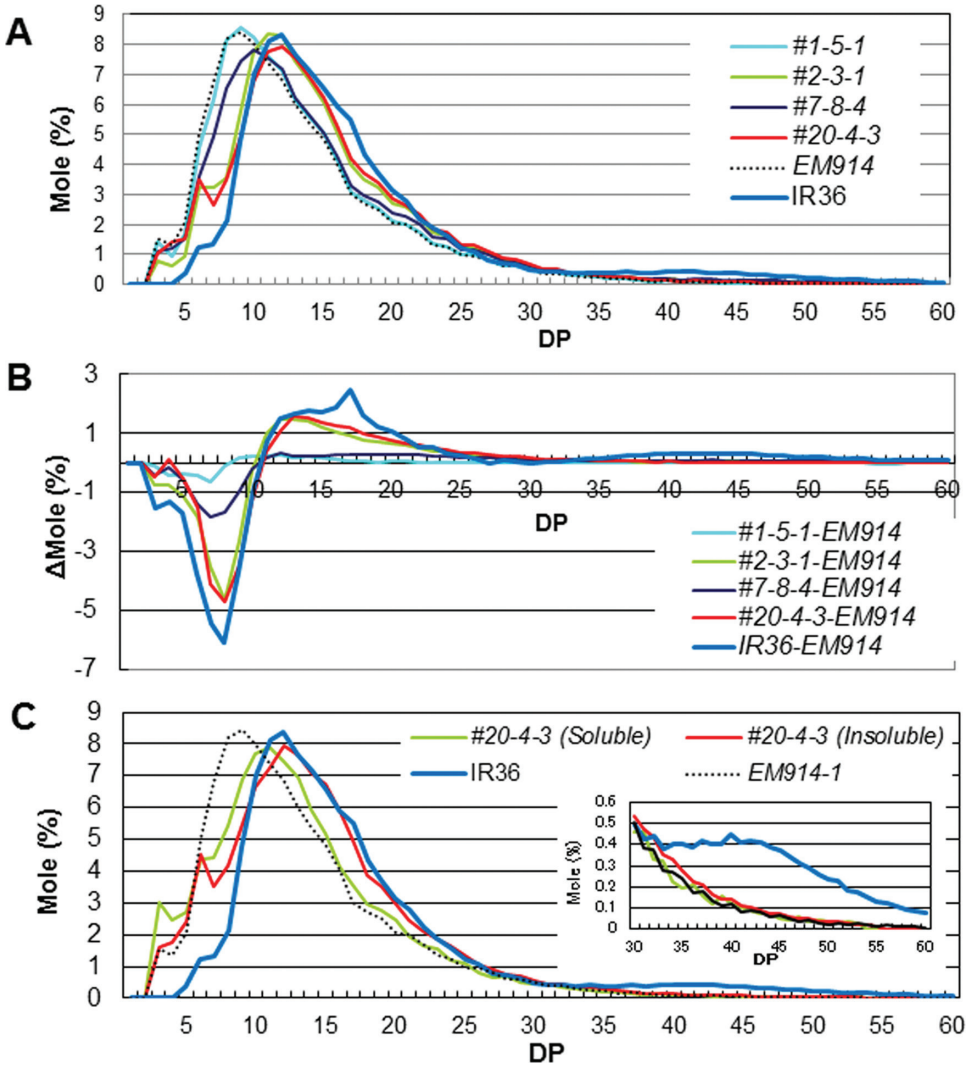

Fig. 4. (A) Chain length distribution patterns of endosperm total (soluble and insoluble) α-glucans in the mature endosperm of transgenic rice lines, *SSIIa*^{I}*/isa1 (#1*, *#2*, *#7*, and *#20*), *EM914*, and the wild type (IR36). (B) Differences in the chain length distribution patterns of amylopectin in the mature endosperm of transgenic rice lines, *SSIIa*^{I}*/isa1* (*#1*, *#2*, *#7*, and *#20*), the wild type (IR36), and the *isa1* mutant, *EM914*. (C) Chain length distribution patterns of soluble (#20 Soluble) and insoluble (#20 Insoluble) α-glucans in the transgenic rice line *#20*, *EM914*, and the wild type (IR36). The inset in C indicates the magnification of the pattern in the range of chains with DP 30–60. The numbers on the plots represent the DP values.

were significantly low in the four lines of *SSIIa*^{I}*/isa1* as well as in *isa1* (Fig. 4A, B; Nakamura *et al*., 1997; Wong *et al*., 2003).

The peaks of DP 3 and 11 of the chain length distribution pattern in the soluble fraction were observed in *#20*, whereas those of DP 6 and 12 were observed in the insoluble fraction in line *#20*, respectively (Fig. 4C). These results mean that the chain length distribution of insoluble α-glucan in line *#20* shifted toward DP 1–3 longer chains when compared with the soluble fraction. This result indicates that the chains of insoluble α-glucans were more elongated by SSIIa^{I}. Consequently, these chains tend to be insoluble in water. The amount of long chains with DP ≥33 in insoluble and soluble α-glucan in line *#20* was much lower than that of the wild-type amylopectin, although a slightly higher amount was observed for insoluble α-glucan compared with soluble α-glucan in line *#20* (Fig. 4C, inset).

The structure of the soluble and insoluble α-glucans in *SSIIa*^{I}*/isa1-#20* was further analysed. Chain length distributions of the α-glucans were examined by HPSEC after fluorescent labelling of the chains with 2-aminopyridine. This method provides data that are complementary to the FCEP method (Hanashiro *et al*., 2011). The HPSEC results are shown in Fig. 5 (left panels) and Table 2. In Fig. 5A and B, the elution profiles by refractive index (RI) detection are shown to allow easier recognition of the differences in the long chain fractions. Fluorescence detection (chromatograms not shown) produced results consistent with those obtained by the FCEP method (Fig. 4). The RI profiles were divided into three fractions as shown in Fig. 5A, namely ELC, long B chain [B(L)], and short B [B(S)] plus A chain, at inflection points commonly observed for normal amylopectins. In Fig. 5A, amylopectin from T65 and phytoglycogen from the congenic *isa1* mutant line (*EM914*) are compared. Major differences

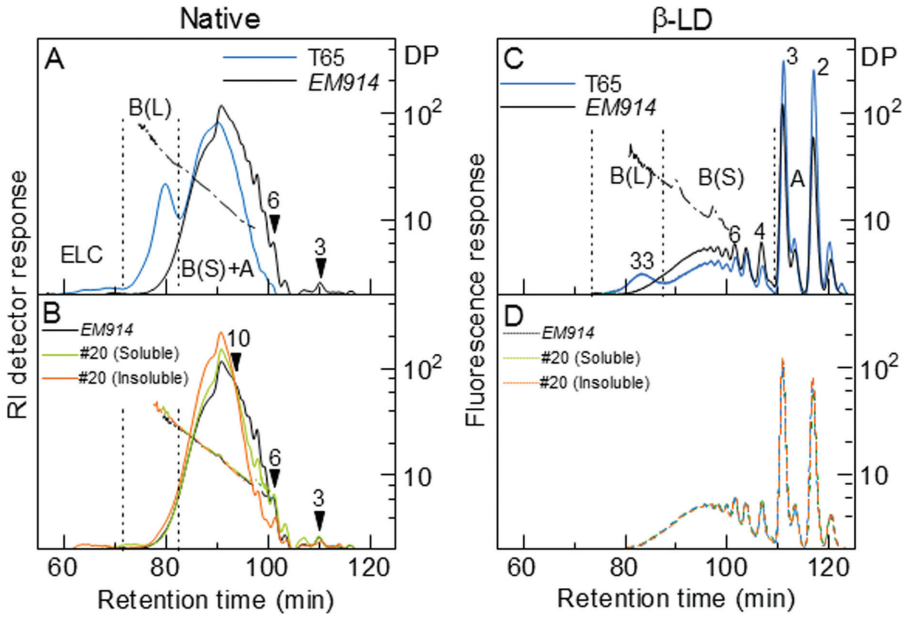

Fig. 5. Chain length distributions of α-glucans and their β-amylase limit dextrins (β-LDs) analysed by HPSEC after labelling with 2-aminopyridine. Solid and dashed line, RI detector (left panels) or fluorescence detector (right panels) response; dash-dot-dash line, DP; number with arrowhead, DP at the specified elution position. These DPs were determined either directly from the ratio of detector responses (RI/fluorescence) or by comparisons with authentic malto-oligosaccharides with known DPs (in the range of DP ≤6). Detector responses are normalized by weight (left panels) or moles (right panels). (A) Amylopectin from the wild-type japonica line T65 and phytoglycogen from the congenic *isa1* mutant line *EM914*. (B) *isa1* phytoglycogen and soluble and insoluble α-glucan of *SSIIa*^{I}*/isa1-#20*. (C) β-LDs of T65 and *EM914*. (D) β-LDs of *EM914* and soluble and insoluble α-glucan of *SSIIa*^{I}*/isa1-#20*.

caused by the loss of ISA1 were: loss of a peak for the B(L) fraction, which corresponds to B_2 or B_3 chains of amylopectin; and an increased amount of short chains eluted at retention times of ≥90 min. These differences indicate that *EM914* phytoglycogen has a higher degree of branching with a greater abundance of shorter unit chains. Additionally, considering the role of long B (B_2 or B_3 of amylopectin with normal cluster structure) chains in the arrangement of clusters in a tandem fashion in an amylopectin molecule, such an organized structure is absent in *EM914* phytoglycogen as compared with normal amylopectin (Nakamura *et al*., 1997; Wong *et al*., 2003). Figure 5B shows that α-glucans from line *#20* endosperm are composed of fewer short chains with DP 3–11 than those of *EM914*. A significant reduction was observed in different DP ranges, including DP 7–11 for the soluble fraction and DP 3–10 for the insoluble fractions in line *#20*. The extent of the reduction of these short chains was larger in the insoluble fractions than in the soluble fraction of line *#20*. In the DP range of ≥12, including the B(L) and ELC fractions, the amount of unit chains was larger in the insoluble fraction than in the soluble fraction of line *#20* or in *EM914* (Fig. 5A, Table 2)

Chain length distributions of the β-amylase limit dextrins (β-LDs) of the α-glucans

Chain length distributions of the β-amylase limit dextrins (β-LDs) of the α-glucans were analysed to examine the effect(s), if any, of the mutations and the introduction of the *SSIIa*^{I} gene on the branched structure of these α-glucans [Fig. 5 (right panels) and Table 3]. Chromatograms on a molar basis (by fluorescence detection) are shown in Fig. 5C and D. With regard to the B chains, the chain length of β-LDs indicates the position of the outermost branch point of the B chain. Similar to the native α-glucans (Fig. 5A), the elution profiles were divided into three fractions [B(L), B(S), and A] according to the characteristic inflection points in the elution profiles (Fig. 5C). Consistent with the results shown in Fig. 5B, the β-LDs of the phytoglycogens of *isa1* and the soluble and insoluble α-glucans of *SSIIa*^{I}*/isa1* did not exhibit a peak near the retention time of 83 min. This peak is typically detected for normal

Table 2. *Chain length distributions of rice α-glucans*[a]

Sample	Fraction		
	ELC	B(L)	B(S)+A
Mole (%)			
T65	<0.1	9.0±0.1	90.9±0.2
EM914	–[b]	1.7±0.2	98.3±0.2
#20 (Soluble)	<0.1	1.7±0.1	98.2±0.1
#20 (Insoluble)	<0.1	2.9±0.2	97.0±0.1
Weight (%)			
T65	2.0±0.1	22.8±0.4	75.2±0.4
EM914	0.6±0.5	4.9±0.2	94.5±0.3
#20 (Soluble)	0.2±0.2	4.9±0.7	94.9±0.8
#20 (Insoluble)	1.1±0.7	7.1±0.7	91.8±0.1

[a] Values are means ±SD (*n*=3 or 5). Each fraction is designated as shown in Fig. 5A. The B(L) and B(S)+A fractions are equivalent to the B_2+B_3 and B_1+A fraction, respectively, of the designation for normal amylopectin.
[b] Not detected.

Table 3. *Chain length distributions of β-LDs of rice α-glucans*[a]

Sample	Fluorescence peak area (%)		
	B (L)	B (S)	A
T65	9.4±0.4	33.9±0.4	56.7±0.5
EM914	4.0±0.1	51.8±0.3	44.2±0.3
#20 (Soluble)	3.9±0.1	51.6±0.4	44.5±0.5
#20 (Insoluble)	4.1±0.1	50.2±0.2*	45.7±0.2*

[a] Values represent means ±SD (n=3 or 4). Each fraction is designated as shown in Fig. 5C. For T65 amylopectin, the B(L) and B(S) fractions are equivalent to the B_2+B_3 and B_1 fraction, respectively, of the designation for normal amylopectins. An asterisk indicates that the values are significantly different between *EM914* and #20 (insoluble and soluble) (by t-test with Bonferroni correction, $P < 0.0167$).

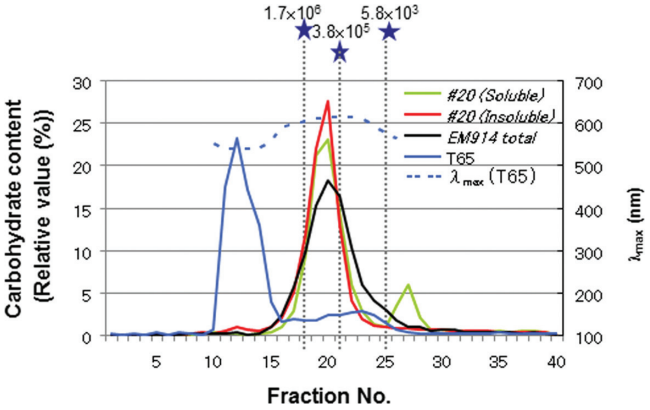

Fig. 6. Size separation of α-glucans of *SSIIa*^{I}*/isa1-#20*, the wild type (T65), and the host *isa1* (*EM914*) by Sephacryl S-1000SF gel filtration chromatography. Solid lines, carbohydrate content by the enzymatic method (left axis); dashed lines, λ_{max} of the T65 starch and iodine complex (right axis); grey dashed lines and stars, commercial pullulan standards with defined average molecular weights.

amylopectin as a peak of long B (B_2 and B_3) chains. In agreement with the indispensable role of isoamylase in constructing the normal cluster structure (Nakamura, 2002; Zeeman *et al*., 2010), the absence of such long B chains in these *isa1* lines implies that a characteristic feature of normal amylopectin, where multiple clusters are connected to each other, is absent in the phytoglycogens and the soluble and insoluble α-glucans from *SSIIa*^{I}*/isa1* transformant *#20*. Figure 5D shows that the β-LDs of α-glucans from *EM914* and its transformant *SSIIa*^{I}*/isa1* (#20 Soluble and Insoluble) contain nearly identical chain length distributions despite the significantly different chain length distributions prior to the exhaustive trimming with β-amylase (Fig. 5B). The amount of each fraction is summarized in Table 3. No significant differences were observed in the amounts of any fractions between the soluble fraction in line *#20* and *EM914*. However, slight differences were observed between the insoluble fraction in line *#20* and the others regarding the amount of the A and B(S) fractions. The α-glucan of the insoluble fraction in line *#20* contained slightly more A chains and concomitantly fewer short B chains.

Molecular size separation of α-glucans of SSIIa^{I}/isa1 *transgenic rice*

To determine the rough molecular weight of the α-glucans in the *SSIIa*^{I}*/isa1* line (*#20*), whole α-glucans (non-digested) were dissolved in 1 N NaOH and used for gel filtration column chromatography (Sephacryl-S1000). Based on the λ_{max} values of the α-glucan–iodine complexes, the fractions containing the majority, if not all, of the amylopectin and amylose of the wild-type starch (T65) eluted in fractions 10–15 and 17–27, respectively (Fig. 6). The molecular weight of amylopectin was much greater than 1.7×10^6, as determined from the pullulan standard, and was estimated to be larger than 10^8 using the HPSEC-MALLS-RI method (Fujita *et al*., 2003; Wong *et al*., 2003). In contrast, the main peak of total α-glucans (almost phytoglycogen) in *EM914* was detected in fraction 20, much smaller than that of the amylopectin (Fig. 6; Nakamura *et al*., 1997). The molecular weight of phytoglycogen was between 3.8×10^5 and 1.7×10^6 based on the pullulan standard. Although the amount of molecules eluted in fractions ≤18 was slightly higher in the insoluble fraction than in the soluble fraction in line *#20*, the vast majority of molecules were eluted in the same range of fractions, around fraction 20, in both cases. Therefore, in terms of average molecular weight and its distribution, the α-glucans of soluble and insoluble fractions in line *#20* are not significantly different. The second peak that appeared at fraction 27 in regards to the soluble fraction was most probably a mixture of small oligo- or monosaccharides.

Crystallinity and granular structure of α-glucans of SSIIa^{I}/isa1 *transgenic rice*

The X-ray diffraction method is commonly used to determine the degree of crystallinity. In this study, crystallinity was estimated by the DSC method by the smaller scale measurement as well as the X-ray diffraction method (Fig. 7). The total α-glucans of *isa1* and *#20* (*EM914* total and line *#20* total) did not show an obvious Δ*H* peak, although an apparent Δ*H* peak at 62 °C was observed in the starch of T65 (Fig. 7A; Wong *et al*., 2003). In contrast, the insoluble α-glucans in line *#20* [#20 (Insoluble)] showed obvious deviation of the measured thermogram from a baseline at ca. 50 °C and 70 °C (Fig. 7A).

Normal rice starches typically show A-type crystallinity. The crystallinity of phytoglycogen in severe *sug-1* mutants, such as *EM914*, was absent (Fig. 7B). In contrast, X-ray diffraction analysis of the insoluble α-glucans in line *#20* revealed a weak B-type diffraction pattern (Fig. 7B). These results suggest that although the crystalline amount may be small, the insoluble α-glucans in line *#20* did exhibit crystallinity. However, the packing of the double helices is quite different between wild-type starch and the insoluble α-glucans in line *#20*.

To analyse whether the chain elongation of phytoglycogen by SSIIa^{I} affects the distinct granular structure of α-glucans, cross-sections of rice seeds were observed by SEM (Fig. 8A). The inner and outer portions of the *isa1* (*EM914*) cross-section did not show a granular structure, although polygonal granules with sharp edges were observed in both portions of the wild type

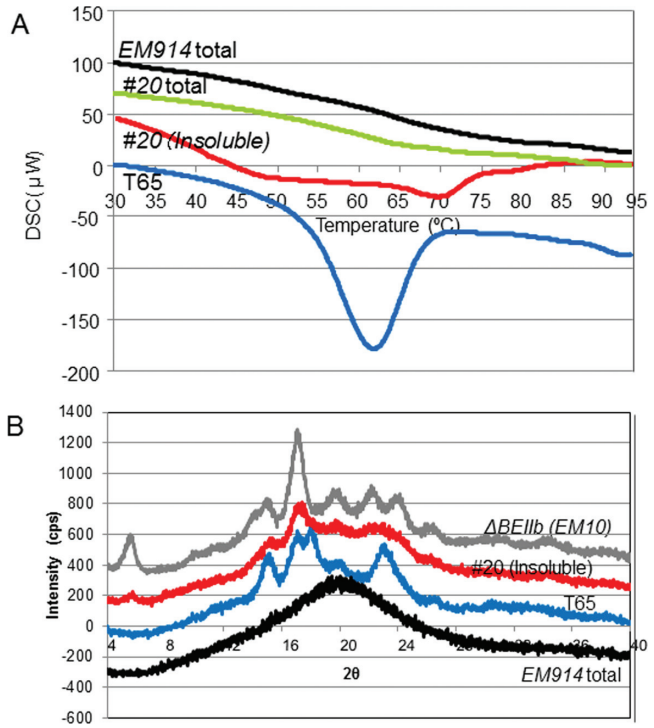

Fig. 7. (A) Differential scanning calorimetric (DSC) curve of rice powder of *SSIIa*^{I}*/isa1-#20* (#20 total), *isa1* (*EM914* total), the wild type (T65), and the insoluble fraction of *#20* (#20 Insoluble). (B) X-ray diffraction pattern of the insoluble fraction of *#20* (#20 Insoluble), rice powder of *EM914* (*EM914* total), and purified starch from T65 and the *be2b* mutant (*EM10*). (This figure is available in colour at *JXB* online.)

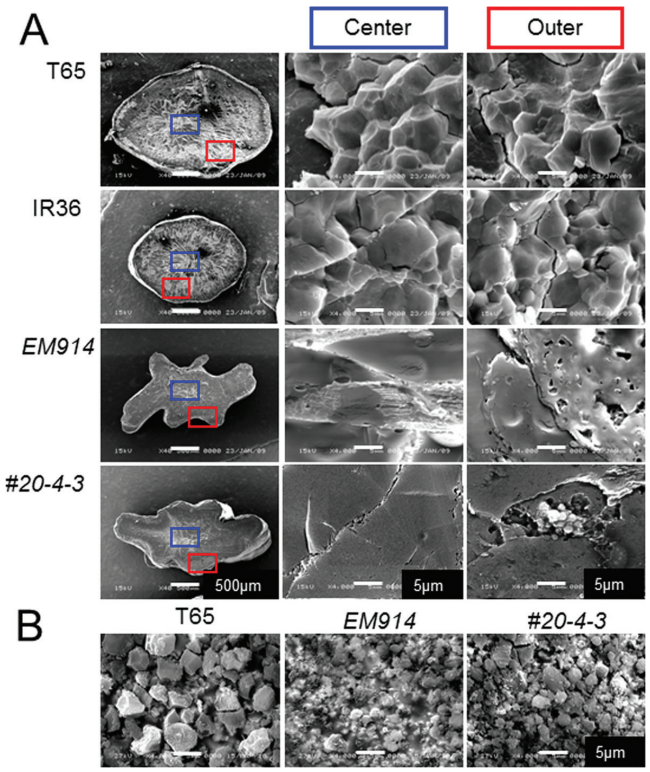

Fig. 8. Scanning electron microscopy (SEM) observations of the centre and outer regions in the cross-sections of maturing seeds (A) and starch granules (B) of transgenic rice (*SSIIa*^{I}*/isa1- #20*), *isa1* (*EM914*), and the wild type (T65 and IR36) purified using Percoll. (This figure is available in colour at *JXB* online.)

(T65 and IR36). On the other hand, some sections of the outer portion of *SSIIa*^{I}*/isa1* contained granular structures that were much smaller than those of the wild type, although no granule structure was detected in the inner portion. For more detailed SEM observations, Percoll-purified granules of the insoluble fraction in line *#20*, the wild type, and *isa1* were examined (Fig. 8B). The granules of *isa1* were much smaller than those of the wild type (Fig. 8B; Wong *et al*., 2003). Interestingly, the Percoll-purified granules from the insoluble fraction in line *#20* appeared as a mixture of large granules of a size equivalent to that of T65 and small granules with a size similar to that of *isa1*.

Discussion

The structure of elongated phytoglycogen by active SSIIa^{I}

The characteristics of the phytoglycogen structure that accumulates in *isa1* (*sug-1*) mutants indicate that it is devoid of B_2 chains and enriched in short chains (Figs 4, 5; Nakamura *et al*., 1997; Wong *et al*., 2003). Compared with amylopectins, the molar ratio of long and short B chains was drastically altered in the phytoglycogens, reduced to less than half and increased by 1.5-fold, respectively (Table 3). These structural changes should result in reduced molecular weight (Fig. 6) and the disruption of the cluster structure typical of normal amylopectin. These results along with previous reports of ISA1-deficient mutant analyses in maize, rice, *Chlamydomonas*, and *Arabidopsis* (James *et al*., 1995; Mouille *et al*., 1996; Nakamura *et al*., 1997; Zeeman *et al*., 1998; Wong *et al*., 2003) strongly suggest that ISA1 function is important for the maintenance of amylopectin structure via removal of improper branch points (Nakamura, 2002; Zeeman *et al*., 2010). Moreover, phytoglycogen is soluble in water and does not exhibit purple coloration with iodine (Table 1, Fig. 3; Nakamura *et al*., 1997). In this study, structural alterations of phytoglycogen (*isa1*) were attempted by introducing SSIIa^{I} (*SSIIa*^{I}*/isa1*) for the production of elongated chains. Since the elongated phytoglycogens exhibit different chain lengths, the structural requirements for the solubility of the α-glucan molecules was investigated at the molecular level, and the crystalline structure was investigated at the granular level.

In the *SSIIa*^{I}*/isa1* lines, in which *SSIIa*^{I} was highly expressed, more than half of the total α-glucans were insoluble (Table 1) and the outer layers of the endosperm tissue of these lines exhibit purple coloration with iodine (Fig. 3). The reason for the localized staining is not clear, but it might be related to the manner of development of the rice endosperm cell. Additionally, the chain length distribution pattern and gel filtration pattern of the *#20* line of *SSIIa*^{I}*/isa1* was shifted to the right by FCEP analysis (Fig. 4A) and to the left by HPSEC analysis of debranched

starch (Fig. 5A), respectively. Notably, the extent of the change in amylopectin chain length distribution among the four lines of *SSIIa*^{I}*/isa1* was positively correlated with the extent of *SSIIa*^{I} expression (Figs 2, 4A, B). These results indicated that the chains are most probably elongated by the active SSIIa^{I}. Meanwhile it cannot be excluded that indirect effects caused by the introduction of SSIIa^{I} on other synthetic enzymes are responsible for the observed chain elongation in glucans from the SSIIa^{I}-expressing lines. The same possibilities similarly apply to the crystallinity observed for the SSIIa^{I}-expressing lines. In contrast to the changes in short chain fractions, the long B_2+ chains with DP ≥33 that connect cluster structures were significantly reduced in the *SSIIa*^{I}*/isa1* lines when compared with normal starches as well as phytoglycogen by FCEP (Fig. 4A, B) and HPSEC analysis (Fig. 5A, B). Additionally, the structure of the β-LDs of *SSIIa*^{I}*/isa1* was similar to that of the β-LDs of phytoglycogen (Fig. 5C, D), indicating that the branch points and branch frequency of the α-glucans in *SSIIa*^{I}*/isa1*-#20 were nearly identical to those of the parental phytoglycogen, although slight differences were observed between insoluble and soluble α-glucans in line *#20* or *EM914* in the amount of the A and B(S) fractions (Table 3). These results strongly suggest that SSIIa^{I} elongates the outer chains of phytoglycogen, but does not affect the location of the branch points (Fig. 9). This also indicates that the chains elongated by SSIIa^{I} are unlikely to be substrates for BEs.

Following the separation of water-suspended α-glucans of line *#20* by 600 *g* centrifugation, further structural characterizations revealed that the outer chains of the insoluble α-glucans of line *#20* (#20 Insoluble) were longer than those of the soluble α-glucans (#20 Soluble) (Figs 4C, 5B). The amount of longer chains with DP ≥33 and ELCs was increased in the insoluble fraction compared with the soluble fraction in line *#20* and the phytoglycogen of the host plant (Figs 4C, 5B, Table 2). In contrast, the molecular weights of the whole α-glucans (not-debranched) were not significantly different between the insoluble and soluble fraction in line *#20* and *isa1* phytoglycogen. Moreover, the insoluble fraction in *#20* exhibited weak B-type crystallinity, which is quite different from the A-type crystallinity of normal rice starches (Fig. 8B). Additionally, the insoluble fraction in *#20* showed a low, but definite, endothermy by DSC measurement (Fig. 8A). Putaux *et al*. (2006) produced modified oyster glycogens whose external chains were extended by recombinant amylosucrase from *Neisseria polysaccharea*. The λ_{max} value of the iodine complex of the products was 614 nm, corresponding to the average chain length of DP 127. The elongated chains formed double-helical segments by intra- and interchain entanglement, resulting in strong B-type crystallinity. In this study, outer chains partially elongated by SSIIa^{I} were able to extend longer than DP 30. This caused weak B-type crystallinity (Hizukuri, 1985) and a small Δ*H* by DSC.

Solubility and crystallinity of α-glucans

The approach of this study was to vary the suite of enzymes present in rice endosperm that are responsible for starch biosynthesis. This was conducted as a means to vary the structure of the α-glucans that are synthesized. The genotypes of the *SSIIa*^{I}*/isa1* transgenic lines used in this study (Table 4) were the same as those of a presumed *sug-1* mutant of *indica* rice, although such a mutant has not been identified to date, or as a maize *su1* mutant line with the exception of the *GBSSI* genotype (typical *japonica* rice cultivars are *gbss1* leaky mutants). Most of the maize *su1* mutant lines contain insoluble, starch-like α-glucans as well as phytoglycogen (Dinges *et al*., 2001). This may be related to the active SSIIa in maize. In contrast, the complete loss of both ISA1 and SSIIa (severe *isa1* mutant lines such as *EM914*) activity impaired the production of insoluble α-glucans.

Quadruple mutants lacking all four DBE proteins (ISA1, 2, 3, and PUL) in *Arabidopsis* are devoid of starch granules and instead accumulate phytoglycogen (Streb *et al*., 2008). On the other hand, the additional loss of the chloroplastic α-amylase AMY3 partially reverts the phenotype of the quadruple DBE

Table 4. *Summary of genotypes, activity levels of SSIIa, ISA, and BEIIb, and phenotypes in lines used in this study and transgenic rice lines containing soluble and insoluble α-glucans*

Lines	References	Genotypes	Activity levels				Dehulled grain weight[a] (mg)	Insoluble α-glucan (%)	Δ*H* by DSC[b] (%)	Crystallinity by X-ray[b] (%)
			SSIIa	ISA (hetero)	ISA (homo)	BEIIb				
T65	This study	WT (*japonica*)	–	+	++	++	19.4	97.4	100	100 (A type)
IR36	This study	WT (*indica*)	+	+	++	++	16.8	97.0	100	100 (A type)
EM914	This study	*isa1* (*japonica*)	–	–	–	+	11.3	3.1	0	0
#20	This study	*OE-SSIIa*^{I}*/*[c]*isa1*	+	–	–	+	9.3	65.6	10	20 (B type)
#G5-1	Fujita *et al*. (2003)	*DR-ISA1/WT*	–	±	±	++	21.0	83.8	50	30 (A type)
#1-1	Tanaka *et al*. (2004)	*OE-BEIIb/be2b*	–	+	++	+++	7.7	89.2	20	10 (A type)
Wx^{a}*:ISA2*	Utsumi *et al*. (2011)	*OE-ISA2/WT*	–	+++	–	+	10.2	92.6	0	5 (?)

[a] Mean of 20 seeds.
[b] Percentage of the wild type.
[c] Background
OE, overexpression; DR, down-regulation.

mutant, restoring starch granules. In maize, in contrast to the single mutant parents, double mutant endosperms affected in both SSIII and ISA2 were starch deficient and accumulated phytoglycogen as shown in ISA1-deficient mutant lines (Lin *et al*., 2012). These previous reports and the results in this study implied that although DBEs are important in starch biosynthesis, other enzymes such as SSIIa in rice, SSIII and SSIIa in maize, and α-amylase in *Arabidopsis* are also indispensable for the distribution of granular and soluble α-glucans.

Transgenic rice lines with increased soluble α-glucans in the endosperm (Table 4) were previously produced by several means: antisense inhibition of *ISA1* (Fujita *et al*., 2003), overexpression of *ISA2* (Utsumi *et al*., 2011), and overexpression of *BEIIb* (Tanaka *et al*., 2004).

In the endosperm of rice line *#G5-1a*, 16.2% soluble α-glucans accumulated. This was mediated by a 94% reduction in ISA1 activity using an antisense transgenic approach (Fujita *et al*., 2003). These soluble α-glucans exhibited a low molecular weight comparable with phytoglycogen and contained numerous short chains as compared with amylopectin. In contrast, the molecular weight of the insoluble α-glucans was similar to that of normal amylopectin. Even so, the short chains with DP ≤9 and long B_2+ chains with around DP ≥40 connecting the amylopectin clusters in soluble α-glucans were significantly increased and decreased, respectively, as compared with amylopectin (Fujita *et al*., 2003).

In the endosperm of rice line *#1-1* (Tanaka *et al*., 2004), 10.8% of the soluble α-glucans accumulated in response to overexpression of the *OsBEIIb* gene in rice *be2b*. The DP ≥40 chains and high molecular weight α-glucans in the soluble fraction of *#1-1* were significantly reduced when compared with the wild type. However, a marked increase in short chains with DP ≤14 was observed (Tanaka *et al*., 2004).

Soluble α-glucans of *Wx^a:ISA2*, a transgenic rice line overexpressing the *OsISA2* gene under the control of the *Wx^a* promoter, were 7- to 8-fold higher relative to the wild type. However, the *OsISA2* repressed lines exhibited nearly the same level as wild-type plants. In contrast, the insoluble α-glucans of *Wx^a:ISA2* did not exhibit any Δ*H* peak by DSC analysis (Utsumi *et al*., 2011).

ISA1, the most critical enzyme for the construction of normal cluster structure (Nakamura, 2002; Zeeman *et al*., 2010), does exist in the transgenic rice lines *#G5-1a* (6% of the wild type) and *#1-1*. In contrast, ISA1 activity in the *#20* line of this study was near zero, as derived from the parent *isa1* (Fig. 2). In the case of *Wx^a:ISA2*, functional ISA1 activity appears to be significantly decreased. Overexpression of *ISA2* and the resulting excess amount of ISA2 protein caused ISA1-2, a non-functional hetero-oligomer, to become dominant over the functional ISA1 homo-oligomer (Utsumi *et al*., 2011). The DSC endothermic peak and the A-type crystallinity (Table 4) of the α-glucans were sustained in *#G5-1a* and *#1-1*, while they were not observed in *SSIIa^l/isa1* (#20 total) and *Wx^a:ISA2*. These results indicate that functional ISA1 activity is indispensable for the crystallinity of α-glucans in rice, through removal of improper branch chains that otherwise interfere with the formation of double helices, even though the outer chains are elongated by SSIIa^I.

The relationships among starch granule structure, water solubility, and molecular structure are still obscure. It is apparent that the normal starch molecular structure can build rigid starch granules, although the reduction of ISA1 activity leads to irregular and small granules (Fig. 8; Boyer *et al*., 1977; Zeeman *et al*., 1998; Burton *et al*., 2002; Fujita *et al*., 2003; Wong *et al*., 2003; Utsumi *et al*., 2011). In contrast, small particles are formed by phytoglycogen and elongated glycogen via recombinant amylosucrase (Fig. 8; Putaux *et al*., 2006). Moreover, the size of the starch granules of even normal starches depends upon the plant species. Further studies are necessary to characterize the regulation of the granule structure of α-glucans.

In summary, this study shed light on relationships among structures, water solubility, and crystallization of plant storage α-glucans (Fig. 9). Phytoglycogen produced in *EM914*, an *isa1* mutant, was present in a highly branched, soluble form with no measurable crystallinity and did not form water-insoluble

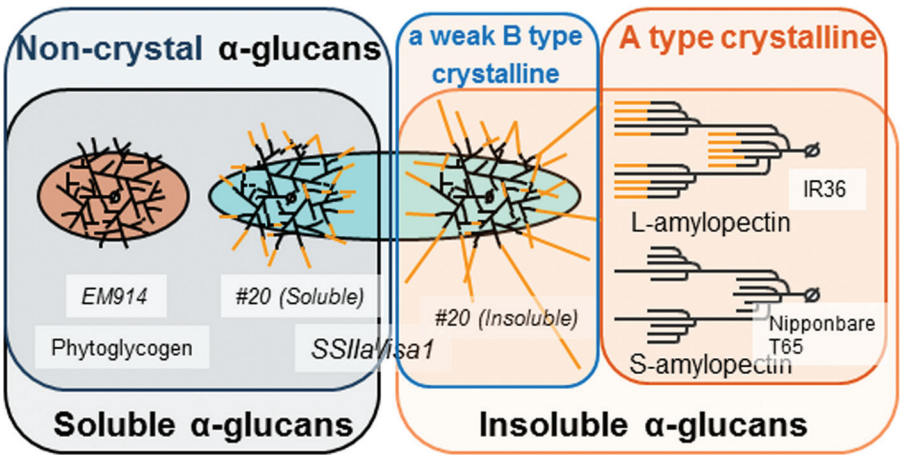

Fig. 9. Schematic representation of the α-glucans in this study, phytoglycogen in *EM914*, soluble and insoluble α-glucan in the transgenic rice line (*#20*), and L- and S-amylopectin in indica and japonica rice, respectively. Insoluble α-glucan in line *#20* is more elongated than the soluble α-glucan and phytoglycogen. Amylopectin exhibits A-type crystallinity, whereas phytoglycogen and α-glucan in *SSIIa^l/isa1* have no (phytoglycogen and soluble α-glucan) or a weak B-type (insoluble α-glucan) crystallinity.

granules. Differences in the chain length of the outer chains in the line *#20* insoluble glucan, which is affected either by the slightly increased amount of relatively long chains and/or by elongation of relatively short chains by ~3 residues, is critically important for whether or not the glucan chains crystallize, and consequently the crystallinity influences the solubility of the glucans. The line *#20* insoluble glucan occurred in endosperm in a small granule form (particle or aggregate? Fig. 8B) but still not in a granular form like normal starch. The degree of branch frequency and branch point location are indispensable for normal crystallinity and granule formation as seen in wild-type starches. Considering solely the occurrence of crystallization, however, they are not necessarily indispensable as shown by the example of the insoluble *SSIIa*^{l}*/isa1* α-glucan with a weak B-type crystallinity in this study. This finding for the *SSIIa*^{l}*/isa1* glucan and elongated glycogen via recombinant amylosucrase (Putaux *et al*., 2006) further indicates that crystallization occurred even in the absence of long B chains, which generally are required to connect cluster units, implying that the arrangement of unit chains in a cluster fashion and/or tandemly connected clusters is not an essential requirement for crystallization itself. The same finding also suggests that crystallization and granule formation are not different reflections of the same phenomena. Crystallization at least seems to be a physical process independent of granule formation, while the former might be a necessary condition for the latter.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Construction of the transgenic rice line, *SSIIa*^{l}*/isa1*.

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