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 (SSIIaI, 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 SSIIaI/isa1 lines. Thus, the α-glucans from the SSIIaI/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 SSIIaI/isa1 insoluble > SSIIaI/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 SSIIaI/isa1 transgenic lines, the unit chains of α-glucans were elongated by SSIIaI, whereas the expression of SSIIaI 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 SSIIaI.

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.

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tissue and substrate specificities. Several SS genes exist in green plants. Among these, the functions of granule-bound starch synthase I (GBSSI), 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). SSII generates DP (degree of polymerization) 8–12 chains from DP 6–7 chains that emerge from the branch point in the A and B2 chain of amylopectin (Fujita et al., 2006, 2008). SSIII(a) functions to elongate the long B2,3 chains that connect multiple clusters of amylopectin (Inouchi et al., 1983; Fulton et al., 2002; Raš et al., 2006; Fujita et al., 2007; Börén 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., 2005a); barley (sex6: Morell et al., 2003), sweet potato (cv. Quick sweet: Katayama et al., 2002; Kitahara et al., 2005), and Arabidopsis (Ass2: 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 B1 chains). The proportion of longer B chains (DP ≥24; B2 and B3) was unchanged in these plant species, except for sugary2 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., 1988; Edwards et al., 1999; Lloyd et al., 1999; Yamamori et al., 2000; Umemoto et al., 2002; Nakamura et al., 2002; 2005a; 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, 2005a).

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 (Sul) gene by transposon tagging of sul 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. Phytoamylopectin was recovered from supermutants using low-speed centrifugation, indicating that phytoamylopectin is a soluble α-glucan. Phytoamylopectin consists of significantly more short chains of DP ≤10 and fewer chains of 11 ≤DP ≤24 when compared with wild-type amylopectin. In phytoamylopectin, the quantity of long chains with DP ≥37 corresponding to B2,3 chains of amylopectin is significantly decreased when compared with amylopectin. Phytoamylopectin 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, with 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 MBC10216 and in some species of Rhodophyta such as Porphyridium purpureum (Nakamura et al., 2005b; Deschamps et al., 2008; Shimomura 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/isa1) exhibiting elongated outer chains of phytoamylopectin. These plants were produced by introducing the active SSIIa gene of indica rice (SSIIa′)
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 (*japonica* cultivars) and IR36 (*indica* cultivars) were included as wild-type plants in this study. A severe type of the sugary-1 (*sug1*) 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 (Sato 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., 2005a) under the control of the rice Whc 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 T0 progeny lines were isolated from the transformation. Three T1 seeds were randomly chosen from each T0 plant. These seeds were independently analysed for endosperm amylopectin chain length distribution using capillary electrophoresis as described below. Five randomly chosen T1 seeds of four T0 transformed lines were grown, and their seeds (T2) were examined for amylopectin chain length distribution. Western blotting was also conducted using SSIIa antisera (Nakamura et al., 2005a). Four homozygous lines (SSIIa/isa1-#1, #2, #7, and #20) were selected and their seeds (T3) 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 glycan (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 antiseraum 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., 2005a).

**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% I2 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 in Tanaka et al. (2004) and Fujita et al. (2003), respectively. Total α-glucans from a transgenic rice line (SSIIa/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/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/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 Shimomaga 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-JSM-5000, 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 °C 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 Coulter, 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 Sephaeryl 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 $\lambda_{\text{max}}$ 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/isa1 transgenic rice lines*

The SSIIa cDNA derived from the *indica* cultivar IR36 (SSIIai; Nakamura et al., 2005a) was introduced into the severe isa1 mutant, EM914 (Nakamura et al., 1997), whose endogenous SSIIa is inactive (Table 4), using the Agrobacterium method. The isoamylase activity related to starch biosynthesis in the developing endosperm (~15 d after flowering) of the transgenic rice lines (SSIIa/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/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 T3 dry seeds of the SSIIa/isa1 lines was conducted using an antiserum raised against SSIIa. This immunoblot confirmed the expression of the introduced SSIIai gene (Fig. 2). SSIIai, 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., 2005a). Faint SSIIa bands in individual T3 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 bound protein fractions.
bound protein fractions (Fig. 2). These results suggest that the introduced SSIIa\(^a\) 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/isa1 transgenic rice lines**

Cross-sections of the mature kernels of SSIIa/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/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\(^a\) 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\(^a\) 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/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\(^a\) gene expression.

\(\alpha\)-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 \(\alpha\)-glucan in the wild type (T65) was 97.4% of the total amount of \(\alpha\)-glucan in the endosperm. In contrast, the amount of insoluble \(\alpha\)-glucan in isa1 (EM914) was only 3.1% (Table 1; Wong et al., 2003). The amount of insoluble \(\alpha\)-glucan in the SSIIa/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\(^a\) gene expression, contained markedly high quantities of insoluble \(\alpha\)-glucans (51.0–65.4%). Lines \#1 and \#7, which exhibited low SSIIa\(^a\) gene expression, had relatively low quantities of insoluble \(\alpha\)-glucans (26.6–34.5%).

### Table 1. Dehulled grain weight, and soluble and insoluble \(\alpha\)-glucan content in dry seeds of transgenic rice and their parents

| Lines     | Dehulled grain weight\(^a\) (mg) | Soluble \(\alpha\)-glucan\(^b\) (mg) | Insoluble \(\alpha\)-glucan\(^b\) (mg) | % of insoluble fraction of total \(\alpha\)-glucan |
|-----------|----------------------------------|------------------------------------|-------------------------------------|-----------------------------------------------|
| Taichung 65 Wild type | 19.4 ± 1.5                      | 0.38 ± 0.15                       | 14.2 ± 0.73                        | 97.4                                          |
| EM914 isa1 | 11.3 ± 0.2                      | 3.80 ± 0.89                       | 0.12 ± 0.06                        | 3.1                                           |
| \#1 SSIIa/isa1 | 8.1 ± 1.0                       | 2.87 ± 0.38                       | 1.04 ± 0.12                        | 26.6                                          |
| \#2 (3) SSIIa/isa1 | 10.6 ± 0.2                      | 3.49 ± 0.43                       | 3.63 ± 0.54                        | 51.0                                          |
| \#2 (4) SSIIa/isa1 | 8.9 ± 1.0                       | 1.65 ± 0.39                       | 2.54 ± 0.23                        | 60.6                                          |
| \#7 SSIIa/isa1 | 7.8 ± 1.3                       | 3.26 ± 0.46                       | 1.72 ± 0.16                        | 34.5                                          |
| \#20 (4) SSIIa/isa1 | 7.3 ± 0.4                       | 1.58 ± 0.53                       | 2.05 ± 0.33                        | 56.5                                          |
| \#20 (8) SSIIa/isa1 | 9.3 ± 1.0                       | 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.

### The fine structure of \(\alpha\)-glucans

To evaluate the effect of SSIIa\(^a\) gene expression on the fine structure of phytoglycogen, the chain length distribution of the isoamylopectins of the total \(\alpha\)-glucans (see the Materials and methods) in the endosperm was analysed by the FCEP method using the SSIIa/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/isa1 when compared with isa1 (Fig. 4B). Of the SSIIa/isa1 lines, \#2 and \#20 exhibited high expression of SSIIa\(^a\) and high quantities of insoluble \(\alpha\)-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/isa1 (\#1, \#2, \#7, and \#20), the host isa1 mutant (EM914), and the wild type (T65 and IR36).](image-url)
were significantly low in the four lines of SSIIa/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 SSIIaI. 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/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 phytyglycogen from the congenic isa1 mutant line (EM914) are compared. Major differences
caused by the loss of ISA1 were: loss of a peak for the B(L) fraction, which corresponds to B2 or B3 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 (B2 or B3 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 α-glucans

Chain length distributions of the α-glucans were analysed to examine the effect(s), if any, of the mutations and the introduction of the SSIIaI 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/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

| Sample     | Fraction     | ELC | B(L)      | B(S)+A     |
|------------|--------------|-----|-----------|------------|
| T65        | <0.1         |     | 9.0 ± 0.1 | 90.9 ± 0.2 |
| EM914      | <0.1         |     | 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 |
| 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 |

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 B2+B3 and B1+A fraction, respectively, of the designation for normal amylopectin.

2 Not detected.
amylpectin as a peak of long B (B2 and B3) 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 amylpectin, where multiple clusters are connected to each other, is absent in the phytoglycogens and the soluble and insoluble α-glucans from SSIIa/isa1 transformant #20. Figure 5D shows that the β-LDs of α-glucans from EM914 and its transformant SSIIa/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-amylose (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/isa1 transgenic rice

To determine the rough molecular weight of the α-glucans in the SSIIa/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 λmax values of the α-glucan–iodine complexes, the fractions containing the majority, if not all, of the amylpectin and amylose of the wild-type starch (T65) eluted in fractions 10–15 and 17–27, respectively (Fig. 6). The molecular weight of amylpectin was much greater than 1.7 × 106, as determined from the pullulan standard, and was estimated to be larger than 108 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 amylpectin (Fig. 6; Nakamura et al., 1997). The molecular weight of phytoglycogen was between 3.8 × 105 and 1.7 × 106 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/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 ΔH peak, although an apparent ΔH 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 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.
Elongated phytoglycogen in rice endosperm (T65 and IR36). On the other hand, some sections of the outer portion of SSIIα 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 SSIIα

The characteristics of the phytoglycogen structure that accumulates in isa1 (sug-1) mutants indicate that it is devoid of B2 chains and enriched in short chains (Figs 4, 5; Nakamura et al., 1997; Wong et al., 2003). Compared with amylepectins, 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 amylepectin. 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 amylepectin 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 SSIIα (SSIIα/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 SSIIα/isa1 lines, in which SSIIα 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 SSIIα/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 amyllopectin chain length distribution among the four lines of SSIIa/isa1 was positively correlated with the extent of SSIIa expression (Figs 2, 4A, B). These results indicated that the chains are most probably elongated by the active SSIIa. Meanwhile it cannot be excluded that indirect effects caused by the introduction of SSIIa on other synthetic enzymes are responsible for the observed chain elongation in glucans from the SSIIa-expressing lines. The same possibilities similarly apply to the crystallinity observed for the SSIIa-expressing lines. In contrast to the changes in short chain fractions, the long B2+ chains with DP ≥33 that connect cluster structures were significantly reduced in the SSIIa/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/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/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 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 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, endotherm by DSC measurement (Fig. 8A). Pataux 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 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/isa1 transgenic lines used in this study (Table 4) were the same as those of a presumed sug-I mutant of indica rice, although such a mutant has not been identified to date, or as a maize sul1 mutant line with the exception of the GBSSI genotype (typical japonica rice cultivars are gbss1 leaky mutants). Most of the maize sul1 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 (mg) | Insoluble α-glucan (%) | Δ_H by DSC (%) | Crystallinity by X-ray (%) |
|--------|------------------------|-----------------|-----------------|---------------------------|------------------------|----------------|---------------------------|
|        |                        | SSIIa (hetero)  | ISA (homo)      |                           |                        |                |                           |
| T65    | This study             | WT (japonica)   | –               | +                         | ++                     | 19.4           | 97.4                      | 100 (A type)             |
| IR36   | This study             | WT (indica)     | +               | +                         | ++                     | 16.8           | 97.0                      | 100 (A type)             |
| EM914  | This study             | isa1 (japonica) | –               | –                         | –                      | 11.3           | 3.1                       | 0                          |
| #20    | This study             | OE-SSIIa/isa1   | –               | –                         | +                      | 9.3            | 65.6                      | 10 (B type)              |
| #G5-1  | Fujita et al. (2003)   | DR-ISA1/WT      | –               | ±                         | ±                      | 21.0           | 83.8                      | 50 (A type)              |
| #1-1   | Tanaka et al. (2004)   | OE-BEIib/be2b   | –               | +                         | ++                     | 7.7            | 89.2                      | 20 (A type)              |
| WxC/ISA2 | Utsumi et al. (2011)   | OE-ISA2/WT     | –               | +++                      | –                      | 10.2           | 92.6                      | 0 (5 (%)                  |

* Mean of 20 seeds.
* Percentage of the wild type.
* Background. OE, overexpression; DR, down-regulation.
However, the OsISA2 repressed lines exhibited nearly the same moter, were 7- to 8-fold higher relative to the wild type. The DP ≥40 chains and OsBEIIb be2b sion of the gene in rice observed (Tanaka et al., 2004). 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 B2+ 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 #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 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 B2+ 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:ISA2, a transgenic rice line overexpressing the OsISA2 gene under the control of the Wxα 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 Wxα:ISA2 did not exhibit any ΔH 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 Wxα: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/isa1 (#20 total) and Wxα: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.

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 amylase (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 granular and soluble α-glucans.

![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-amylase 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/isa1 have no (phytoglycogen and soluble α-glucan) or a weak B-type (insoluble α-glucan) crystallinity.](image-url)
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/isa1 α-glucan with a weak B-type crystallinity in this study. This finding for the SSIIa/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/isa1.

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References
Ball SG, Morell MK. 2003. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. Annual Review of Plant Biology 54, 207–233.

Borén M, Glaring MA, Ghebremedhin H, Olsson H, Blennow A, Jansson C. 2008. Molecular and physicochemical characterization of the high-amylose barley mutant Arno1. Journal of Cereal Science 47, 79–89.

Boyer CD, Daniels RR, Shannon JC. 1977. Starch granules (amyloplast) development in endosperm of several Zea mays L. genotypes affecting kernel polysaccharides. American Journal of Botany 64, 50–56.

Burton RA, Jenner H, Carrangis L, et al. 2002. Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. The Plant Journal 31, 97–112.

Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL, Smith AM. 1998. Mutations in the gene encoding starch synthase II profoundly alter amyllopectin structure in pea embryos. The Plant Cell 10, 413–426.

Deschamps P, Colleoni C, Nakamura Y, et al. 2008. Metabolic symbiosis and the birth of the plant kingdom. Molecular Biology and Evolution 25, 536–548.

Dinges JR, Colleoni C, Myers AM, James MG. 2001. Molecular structure of three mutations at the maize sugary1 locus and their allelic-specific phenotypic effects. Plant Physiology 125, 1406–1418.

Edwards A, Fulton DC, Hylton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith AM. 1999. A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. The Plant Journal 17, 251–261.

Fujita N, Goto S, Yoshida M, Suzuki E, Nakamura Y. 2008. The function of rice starch synthase I expressed in E. coli. Journal of Applied Glycoscience 55, 167–172.

Fujita N, Hasegawa H, Taia T. 2001. The isolation and characterization of a waxy mutant of diploid wheat (Triticum monococcum L.). Plant Science 160, 595–602.

Fujita N, Kubo A, Francisco PB Jr, Nakakita M, Harada K, Minaka N, Nakamura Y. 1999. Purification, characterization, and cDNA structure of isoamylase from developing endosperm of rice. Planta 206, 283–293.

Fujita N, Kubo A, Suh S-D, Wong K-S, Jane J-L, Ozawa K, Takaia F, Inaba Y, Nakamura Y. 2003. Antisense inhibition of isoamylase alters the structure of amyllopectin and the physicochemical properties of starch in rice endosperm. Plant and Cell Physiology 44, 607–618.

Fujita N, Toyosawa Y, Utsumi Y, et al. 2009. Characterization of PUL-deficient mutants of rice (Oryza sativa L.) and the function of PUL on the starch biosynthesis in the rice endosperm. Journal of Experimental Botany 60, 1009–1023.

Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, Nakamura Y. 2006. Function and characterization of starch synthase I using mutants in rice. Plant Physiology 140, 1070–1084.

Fujita N, Yoshida M, Kondo T, et al. 2007. Characterization of SSIIa-deficient mutants of rice: the function of SSIIa and pleiotropic effects by SSIIa deficiency in the rice endosperm. Plant Physiology 144, 2009–2023.

Fulton DC, Edwards A, Pillung E, et al. 2002. Role of granule-bound starch synthase in determination of amyllopectin structure and starch granule morphology in potato. Journal of Biological Chemistry 277, 10834–10841.

Gidley MJ, Bulpin PV. 1987. Crystallisation of malt-oligosaccharides as models of the crystalline from of starch: minimum chain-length
requirement for the formation of double helices. *Carbohydrate Research* **161**, 291–300.

Hanashiro I, Higuchi T, Aihsara S, Nakamura Y, Fujita N. 2011. Structure of starches from rice mutants deficient in the starch synthase isozyme SSI or SSI1. *Biocromolecules** **12**, 1621–1628.

Hanashiro I, Itoh K, Kuratomi Y, Yamazaki M, Igarashi T, Matsugasak J, Takeda Y. 2008. Granule-bound starch synthase I is responsible for biosynthesis of extra-long units chains of amylopectin in rice. *Plant and Cell Physiology** **49**, 925–933.

Hizukuri S. 1985. Relationship between the distribution of the chain length of amylopectin and crystalline structure of starch granules. *Carbohydrate Research** **141**, 295–306.

Hood EE, Gelvin SB, Melchers LS, Hoekema A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research** **2**, 208–218.

Inouchi N, Glover DV, Takaya T, Fuwa H. 1983. Development changes in fine structure of starches of several endosperm mutants of maize. *Starch/Stärke** **35**, 371–376.

James MG, Robertson DS, Myers AM. 1995. Characterization of the maize gene sugary 1, a determinant of starch composition in kernels. *The Plant Cell** **7**, 417–429.

Katayama K, Komae K, Koyama K, Kato T, Tamiya S, Komaki K. 2002. New sweet potato line having low gelatinization temperature and altered starch structure. *Starch/Stärke** **54**, 51–57.

Kitahara K, Fukunaga S, Katayama K, Takahata Y, Nakazawa Y, Yoshinaga M, Suganuma T. 2005. Physicochemical properties of sweetpotato starches with different gelatinization temperatures. *Starch/Stärke** **57**, 473–479.

Kubo A, Fujita N, Harada K, Matsuda T, Satoh H, Nakamura Y. 1999. The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiology** **121**, 399–409.

Kubo A, Rahman S, Utsumi Y, et al. 2005. Complementation of sugary-1 phenotype in rice endosperm with the wheat isoamylase t gene supports a direct role for isoamylase t in amylopectin biosynthesis. *Plant Physiology** **137**, 43–56.

Lin Q, Huang B, Zhang M, Zhang X, Rivenbak J, Lappe RL, James MG, Myers AM, Hennen–Bierwagen TA. 2012. Functional interactions between starch synthase I1 isoform and isomaltooligosaccharide-type starch-debranching enzyme in maize endosperm. *Plant Physiology** **158**, 679–692.

Lloyd JR, Landschutze, V, Kossmann J. 1999. Simultaneous antiserum inhibition of two starch-synthase isoforms in potato tubers leads to accumulation of grossly modified amylopectin. *Biochemical Journal** **338**, 515–521.

Morell MK, Kosar–Hashemi B, Cmiel M, Samuel MS, Chandler P, Rahman S, Buleon A, Batay LL, Li Z. 2003. Barley sex1 mutants lack starch synthase I1a activity and contain a starch with novel properties. *The Plant Journal** **34**, 173–185.

Mouille G, Maddelein M–L, Libessart N, Talaga P, Decq A, Delrue B, Ball S. 1996. Pre-amylopectin processing: a mandatory step for starch biosynthesis in plants. *The Plant Cell** **8**, 1353–1366.

Myers AM, Morell MK, James MG, Ball SG. 2000. Recent progress toward understanding biosynthesis of the amylopectin crystal. *Plant Physiology** **122**, 989–997.

Nakamura Y. 2002. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant and Cell Physiology** **43**, 718–725.

Nakamura Y, Francisco PB Jr, Hosaka Y, Sato A, Sawada T, Kubo A, Fujita N. 2005a. Essential amino acids of starch synthase I1a differentiate amylopectin structure and starch quality between japonica and indica rice varieties. *Plant Molecular Biology** **58**, 213–227.

Nakamura Y, Kubo A, Shimamune T, Matsuda T, Harada K, Satoh H. 1997. Correlation between activities of starch debranching enzyme and α-polyglucan structure in endosperms of sugary-1 mutants of rice. *The Plant Journal** **12**, 143–153.

Nakamura Y, Miyachi S. 1982. Effect of temperature on starch degradation in *Chlorella vulgaris* 11h cells. *Plant and Cell Physiology** **31**, 303–309.

Nakamura Y, Sakurai A, Inaba Y, Kimura K, Iwasawa N, Nagamine T. 2002. The fine structure of amylopectin in endosperm from Asian cultivated rice can be largely classified into two classes. *Starch** **54**, 117–131.

Nakamura Y, Takahashi J–I, Sakurai A, et al. 2005b. Some cyanobacteria synthesize semi-amylopectin type α-polyglucans instead of glycogen. *Plant and Cell Physiology** **46**, 539–545.

Nakamura Y, Unemoto T, Ogata N, Kuboki Y, Yano M, Sasaki T. 1996. Starch debranching enzyme (R-enzyme or pullulanase) from developing rice endosperm: purification, cDNA and chromosomal localization of the gene. *Planta** **199**, 209–218.

Nishi A, Nakamura Y, Tanaka N, Satoh H. 2001. Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. *Plant Physiology** **127**, 459–472.

O'Shea MG Morell MK. 1996. High resolution slab gel electrophoresis of 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) tagged oligosaccharides using a DNA sequencer. *Electrophoresis** **17**, 681–688.

Pan D, Nelson OE Jr. 1984. A debranching enzyme deficiency in endosperms of the sugary1 mutants of maize. *Plant Physiology** **74**, 324–328.

Putaux J–L, Potocki–Véronèse G, Remaud–Simeon M, Buleon A. 2006. α-d-Glucan-based dendritic nanoparticles prepared by in vitro enzymatic chain extension of glycogen. *Biocromolecules** **7**, 1720–1728.

Ral J–P, Colleoni C, Wattebled F, et al. 2006. Circadian clock regulation of starch metabolism establishes GBSSI as a major contributor to amylopectin synthesis in *Chlamydomonas reinhardtii*. *Plant Physiology** **142**, 305–317.

Sano Y. 1984. Differential regulation of waxy gene expression in rice endosperm. *Theoretical and Applied Genetics** **68**, 467–473.

Satoh H, Omura T. 1979. Induction of mutation by treatment of fertilized egg cell with 2-nitroso-N-methyl-N-nitrosourea in rice. *Journal of the Faculty of Agriculture, Kyusyu University** **24**, 165–174.

Shimonaga T, Konishi M, Oyama Y, et al. 2008. Variation in storage α-glucans of the Porphyridiales (Rhodophyta). *Plant and Cell Physiology** **49**, 103–116.

Smith AM, Denyer K, Martin C. 1997. The synthesis of the starch granule. *Annual Review of Plant and Molecular Biology** **48**, 67–87.

Elongated phytoglycogen in rice endosperm
Streb S, Delatte T, Umhang M, Eicke S, Schorderet M, Reinhardt D, Zeeman SC. 2008. Starch granule biosynthesis in Arabidopsis is abolished by removal of all debranching enzymes but restored by the subsequent removal of an endoamylase. *The Plant Cell* **20**, 3448–3466.

Takeda Y, Hizukuri S, Juliano BO. 1987. Structures of rice amylopectins with low and high affinities for iodine. *Carbohydrate Research* **168**, 79–88.

Takeda Y, Preiss J. 1993. Structure of B90 (sugary) and W64A (normal) maize starches. *Carbohydrate Research* **240**, 265–275.

Tanaka N, Fujita N, Nishi A, Satoh H, Hosaka Y, Ugaki M, Kasawaki S, Nakamura Y. 2004. The structure of starch can be manipulated by changing the expression levels of starch branching enzyme IIb in rice endosperm. *Plant Biotechnology Journal* **2**, 507–516.

Umemoto T, Yano M, Satoh H, Shomura A, Nakamura Y. 2002. Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. *Theoretical and Applied Genetics* **104**, 1–8.

Utsumi Y, Utsumi C, Sawada T, Fujita N, Nakamura Y. 2011. Functional diversity of isoamylase (ISA) oligomers: the ISA1 homo-oligomer is essential for amylopectin biosynthesis in rice endosperm. *Plant Physiology* **156**, 61–77.

Wong K–S, Kubo A, Jane J–L, Harada K, Satoh H, Nakamura Y. 2003. Structures and properties of amylopectin and phytoglycogen in the endosperm of sugary-1 mutants of rice. *Journal of Cereal Science* **37**, 139–149.

Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T. 2000. Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. *Theoretical and Applied Genetics* **101**, 21–29.

Yamanouchi H, Nakamura Y. 1992. Organ specificity of isoforms of starch branching enzyme (Q-enzyme) in rice. *Plant and Cell Physiology* **33**, 985–991.

Zeeman SC, Kossmann J, Smith AM. 2010. Starch: its metabolism, evolution, and biotechnological modification in plants. *Annual Review of Plant Biology* **61**, 209–234.

Zeeman SC, Umemoto T, Lue W–L, Au–Yeung P, Martin C, Smith AM, Chen J. 1998. A mutant of *Arabidopsis* lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *The Plant Cell* **10**, 1699–1711.

Zhang X, Colleoni C, Ratushna V, Sirghiè–Colleoni M, James MG, Myers AM. 2004. Molecular characterization demonstrates that the *Zea mays* gene sugary2 codes for the starch synthase isoform SSIIa. *Plant Molecular Biology* **54**, 865–879.

Zhang X, Szydlowski N, Delvallé D, D’Hulst C, James MG, Myers AM. 2008. Overlapping functions of the starch synthase SSII and SSIII in amylopectin biosynthesis in *Arabidopsis*. *BMC Plant Biology* **8**, 96–113.