Starch Biosynthetic Protein Complex Formation in Rice ss2a be2b (+) Double Mutant Diffs from Their Parental Single Mutants

(Received December 13, 2021; Accepted February 28, 2022) (J-STAGE Advance Published Date: March 5, 2022)

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Abstract: Amylopectin, which consists of highly branched glucose polymers, is a major component of starch. Biochemical processes that regulate the elongation of glucose polymers and the generation and removal of glucose branches are essential for determining the properties of starch. Starch synthases (SSs) and branching enzyme (BE) mainly form complexes consisting of SSI, SSIIa, and BEIIb during endosperm development. Loss of BEIIb in rice is complemented by BEIIa, but the compensatory effects differ depending on the presence or absence of inactive BEIIb. To better understand these compensatory mechanisms, ss2a be2b (+) double mutant, which possessed truncated inactive SSIIa and inactive BEIIb, were analyzed. Soluble proteins separated by gel filtration chromatography showed that SSIIa and BEIIb proteins in the wild-type exhibited a broad range of elution patterns and only small amounts were detected in high molecular mass fractions. In contrast, most of truncated inactive SSIIa and inactive BEIIb from ss2a be2b (+) were found in high molecular mass fractions, and the SSI-SSIIa-BEIIb trimeric protein complex found in the wild-type was likely absent in ss2a be2b (+). Those SSI and BEIIb proteins in high molecular mass fractions in ss2a be2b (+) were also identified by mass spectrometry. Parental ss2a single mutant had negligible amounts of SSIIa suggesting that the truncated inactive SSIIa was recruited to high-molecular mass complexes in the presence of inactive BEIIb in ss2a be2b (+) double mutant. In addition, SSIVb might be involved in the formation of alternative protein complexes with < 300 kDa in ss2a be2b (+).

Key words: amylopectin, protein complex, rice starch biosynthesis, starch branching enzyme, starch synthase

INTRODUCTION

Starch is synthesized as a product of photosynthesis and stored in cereal grains, such as rice, maize, and wheat as well as in legumes and potato tubers. Starch is not only consumed as food, but also used for various industrial applications because of its unique characteristics. Starch consists of glucose polymers with essentially linear amylose and highly branched amylopectin. Amylopectin possesses a tandem glucose polymers with essentially linear amylose and highly branched amylopectin. For example, SSIIIa synthesizes α-1,4 linked glucans with a degree of polymerization (DP) > 30. On the other hand, BEI generates α-1,6 branched intermediate chains with DP 12 to 24 in the amorphous regions. BEIIb generates α-1,6 linked short branches with DP 6 and 7 onto the branches synthesized by BEI. The branches generated by BEIIb are elongated further through the activity of SSI to DP 8 to 12. Finally, additional branch lengthening to DP 12 to 24 is facilitated by active-type SSIIa in typical indica rice cultivars. These sequential reactions allow amylopectin to attain its precise cluster structure.²

It has long been assumed that these starch biosynthetic enzymes form protein complexes during starch synthesis.³⁴ The formation of protein complexes was first revealed in developing seeds of wheat and maize by Tetlow et al.,³ and Tetlow et al.³ Subsequent studies showed that these protein complexes consist of SSI, SSIIa, and BEIIb in cereals such as maize³⁸ and rice.³ The three protein complex components are involved in the formation of short and intermediate chains of amylopectin, suggesting that the trimeric protein complex of SSI-SSIIa-BEIIb should be important for amylopectin biosynthesis.¹⁰ⁱ³¹⁴
The formation of protein complexes among starch biosynthetic enzymes is shown to be dependent on post-translationally altered protein modifications. For instance, phosphorylation of BEIIb and SSIIa in maize triggers the formation of trimeric protein complexes from the monomeric state and from the high molecular mass protein complexes, where BEIIb indirectly associates with SSI via SSIIa. However, the critical phosphorylation sites of BEIIb and SSIIa in maize are not considered in rice. Furthermore, regulatory mechanisms by which starch biosynthetic enzyme complexes are formed in rice remain unclear.

Protein complex formation of starch biosynthetic enzymes in single mutants of rice lacking SSIIa or expressing inactive BEIIb have been analyzed. Such analyses revealed that other isozymes compensate for the absence of SSIIa or BEIIb. For example, the ss2a rice mutant, EM204, has a guanine to adenine mutation in the last nucleotide of the fifth intron. This single nucleotide substitution in the fifth intron causes the non-translation of the sixth exon of the SS2a transcript resulting in a truncated inactive SSIIa, which is degraded subsequently. The observation of protein complex formation in the ss2a mutant suggests SSI likely substituted for SSIIa and formed an alternative protein complex with BEIIb.

There are other examples demonstrating that the absence of a specific starch biosynthetic enzyme can be compensated for by other isozymes. For instance, in developing seeds of the be2b rice mutant (ssg3), which expresses inactive BEIIb, increased levels of high molecular mass protein complexes that were greater than 700 kDa. Such high molecular mass protein complexes did not form in the be2b null mutant EM10. Immunoprecipitation analyses revealed that high molecular mass protein complexes in the endosperm consisted of SSI, SSIIa, BEI and inactive BEIIb. It was proposed that inactive BEIIb could recruit other starch biosynthetic enzymes to form large > 700 kDa protein complexes. In addition, BEIIb compensated for inactive BEIIb by forming trimeric SSI-SSIIa-BEIIa complexes. To further analyze the formation of alternative trimeric protein complexes, double mutant lines were generated by crossing the ss2a mutant with the be2b mutant, which expresses an inactive BEIIb. Another double mutant line was made by crossing ss2a with the null be2b mutant. Mutants expressing inactive BEIIb are designated as be2b (+) while the null mutants are referred to as be2b (-). The structure and physicochemical properties of starch in the different genotypes were examined. Both ss2a be2b (-) and ss2a be2b (+) double mutants were influenced more by the loss of BEIIb than the loss of SSIIa. This conclusion was supported by the observation that the starch structure of the double mutants most closely resembled that of the be2b single mutants. In the starch of the double mutants and be2b single mutants, a reduction in the amount of long amylopectin chains and an increase of long amylopectin chains were found relative to the wild-type. This change in amylopectin structure led to an increase in gelatinization temperature in the starch of the ss2a be2b (-) and ss2a be2b (+) double mutant lines. Furthermore, amylose content was higher in the ss2a be2b (-) and ss2a be2b (+) double mutants than those in the wild-type and their parental single mutant lines. Amylose content and relative starch crystallinity are often negatively correlated. However, the starch of the ss2a be2b (-) and ss2a be2b (+) double mutant lines showed higher crystallinity despite having high amylose content. These findings suggest that amylose may have formed double helices with the long chains of amylopectin in ss2a be2b (-) and ss2a be2b (+) double mutant lines resulting in increased crystallinity.

In the present study, protein complex formation in developing endosperm of ss2a be2b (+) double mutant was analyzed to reveal how protein complex compositions are altered and compensated in the absence of two major enzymes responsible for amylopectin biosynthesis.

**MATERIALS AND METHODS**

**Plant materials.** ss2a be2b (+) double mutant (#1522) were generated by crossing a ss2a mutant (EM204) with a be2b (+) mutant (ssg3) expressing an inactive BEIIb. The parental cultivars Kinmaze, the parent of EM204, and Nipponbare, the parent of ssg3, and IR36, an indica cultivar with active-type SSIIa, were used as controls (Table 1). All rice plants were grown in an experimental paddy field at the Akita Prefecture University under natural conditions during the summer season.

**Microscopic observation of mature rice seeds.** Thin sections of mature rice endosperm were stained with iodine and observed by light microscopy, which were essentially the same as described by Matsushima et al.

**Preparation of protein extracts from developing endosperm and gel filtration chromatography.** Samples used for native-PAGE activity staining were extracted from three developing endosperms at 10 to 15 days after flowering following the methods of Miura et al.

A 700 mg aliquot of developing endosperms was extracted and fractionated by gel filtration chromatography using Superdex 200 resin packed in a XK16 column (16/400, GE Healthcare, Chicago, IL, USA) according to Crofts et al. Western blotting was performed as described previously.

**Native-PAGE activity staining.** Gel filtration chromatography was used to separate the soluble proteins extracted from developing seeds. The eluted fractions were then subjected to Native-PAGE activity staining.

Native-PAGE activity staining of SS and debranching enzymes was performed according to Miura et al. BE activity staining for BEIIb was performed using 7.5 % (w/v) acrylamide native-PAGE gels containing 0.0001 % oyster glycogen. SS activity staining was performed using 7.5 %

| Table 1. Genotype of rice lines used in this study. |
|-----------------------------------------------|
| Lines | Genotype | Background |
|-------|-----------|------------|
| #1522 | ss2a be2b (+) | Kin/Nip |
| EM204 | ss2a | Kin |
| ssg3 | be2b (+) | Nip |
| Kinmaze (Kin) | Wild-type | Kin |
| Nipponbare (Nip) | Wild-type | Nip |
(w/v) acrylamide native-PAGE gel to detect SSI and SSIIIa, while 9 % (w/v) acrylamide native-PAGE gel was used to detect SSI and SSIIa.

**Identification of proteins eluted from the high molecular mass fractions of gel filtration chromatography.** High molecular mass fractions from endosperm extracts of ss2a be2b (+) double mutant and wild-type that eluted in fractions 2 and 3 of the gel filtration chromatography were subjected to SDS-PAGE and Western blotting. The molecular mass of fractions 2 and 3 were > 700 kDa. The SDS-PAGE gel that corresponded to the Western blot signal of SSIIa was excised into five pieces. The extracted gel pieces were treated with trypsin and identified by LC-MS/MS as described by Crofts et al. 17)

**Co-immunoprecipitation.** Co-immunoprecipitation was performed as described by Crofts et al. 13, 15 Briefly, soluble proteins were extracted using 3 volumes of buffer containing 10 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 1 mM DTT, and 10 μL/mL plant protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO, USA). Starch was removed by centrifugation. 800 μL each of supernatant was mixed with 100 μL of isozyme specific antibodies, or pre-immune serum as a control. 100 μL of 50 % slurry of protein A Sepharose resin (Sigma-Aldrich Co.) was added and incubated. After extensive washing, proteins were eluted by boiling in 150 μL of SDS-Sample buffer containing 33 mM Tris-HCl, pH 6.8, 3.3 % SDS, 4 % β-mercaptoethanol, 6.6 % (v/v) glycerol, and 50 mM DTT. Supernatant was analyzed by Western blotting.

**RESULTS AND DISCUSSION**

**Seed traits of ss2a be2b (+).**

It was previously shown by scanning electron microscopy that seeds of ss2a be2b (+) were packed with round starch granules that varied in size. 19) The seed weight of ss2a be2b (+) was heavier than that of the parental single be2b (+) mutant, and lighter than that of the parental ss2a mutant. 19)

In the present study, thin sections of mature seeds were stained with iodine and observed by light microscopy (Fig. 1). Polygonal starch granules were fully packaged within the amyloplasts of two wild-type lines (Fig. 1). By contrast, the ss2a be2b (+) double mutant and the parental be2b (+) single mutant had round starch granules of varying sizes (Fig. 1). The large starch granules were consisted with aggregates of several starch granules. These round starch granules are characteristic of be2b and also observed in other double mutant lines lacking BEIIb. 19) Seeds of ss2a single mutant had polygonal starch granules that were similar to those observed in the wild-type. The ss2a mutant also had small amounts of round starch granules (Fig. 1).

**Pleiotropic effects of ss2a be2b (+) on other starch biosynthetic enzymes.**

To verify whether all the rice lines used in this study had the expected SSIIa and BEIIb expression and activity patterns, native-PAGE activity staining and Western blotting were performed on endosperm extracts of ss2a be2b (+) mutant, their parental lines and wild-type indica rice, IR36, which served as a positive control for SSIIa activity (Figs. 2, 3, and S1; see J. Appl. Glycosci. Web site). Native-PAGE activity staining and Western blotting were also performed to reveal how other starch biosynthetic enzymes are affected in ss2a be2b (+) and whether their affinity for the starch granules was altered.

Native-PAGE activity staining of SSIIa confirmed the absence of SSIIa activity in ss2a be2b (+) and ss2a (Fig. 2A). SSIIa activity was detected in the corresponding wild-type indica cultivar, IR36. By contrast, SSIIa activity in the japonica cultivars, Kinmaze and ssg3, was below the detection limit of the native-PAGE activity staining protocol. These observations were consistent with previous studies showing that SSIIa in japonica rice had only 10 % of SSIIa from indica rice (Fig. 2A). 16) The absence of BEIIb activity in ss2a be2b (+) and be2b (+) was also confirmed (Fig. 2B). SSII activities in ss2a be2b (+) and be2b (+) were lower than those in other genotypes (Figs. 2A, C, and S1; see J. Appl. Glycosci. Web site), which is consistent with the results of Nishi et al. 21) The mobility of SSI and pullulanase (PUL) in indica rice was larger than those in other japonica lines because of amino acid substitution described in a study by Chen and Bao. 25) The activities of other starch biosynthetic

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**Fig. 1.** Thin sections of mature endosperm in various genotypes, stained with iodine, and analyzed by light microscopy. Bars are 20 μm.
enzymes, such as BEI, PUL, isoamylase 1 (ISA1), and plastidial phosphorylase 1 (Pho1), were similar in ss2a be2b (+) to those in other genotypes (Fig. 2). The activity band above the ISA1 is derived from BEIIb and was absent in be2b (+) and ss2a be2b (+) (Fig. 2D).

SP, LBP, and TBP were analyzed by Western blotting (Fig. 3). The amounts of SSI were lower in the SP fractions, but higher in the TBP fractions of be2b (+), ss2a be2b (+), and indica rice than those in wild-type japonica rice and ss2a (Fig. 3). SSIIa was absent in the SP and TBP fractions, and only a faint SSIIa signal was detected in the LBP of ss2a. By contrast, SSIIa proteins were present in the SP fractions of ss2a be2b (+) (Fig. 3A). As expected, the size of the truncated SSIIa protein in ss2a be2b (+) was slightly smaller than that of the wild-type (Fig. 3). SSIIa was not detected in the TBP fractions of wild-type japonica rice, ss2a, and ss2a be2b (+), but was present in the TBP fractions of be2b (+) and indica rice. These results are consistent with the studies of Itoh et al., Crofts et al., and Crofts et al.. SSIVb was mostly present in the SP fractions (Fig. 3). The amount of BEIIb in the SP fraction was similar for all genotypes. This observation indicates that the expression levels of BEIIb protein in be2b (+) and ss2a be2b (+) were not affected by the BEIIb mutation (Fig. 3A). The amounts of all analyzed starch biosynthetic enzymes in the LBP fraction of be2b (+) were greater than those of other genotypes (Fig. 3B). Furthermore, the amount of BEI and BEIIa in the TBP fractions were greater in be2b (+) and ss2a be2b (+) than other genotypes (Fig. 3C).

Active-type SSIIa is responsible for the formation of SSI-SSIIa-BEIIb trimeric protein complexes, and also important for the binding of these proteins to the starch granules in maize. When active-type SSIIa was present in the wild-type indica rice, SSI and BEIIb strongly associated with the starch granules (Fig. 3C). By contrast, in the absence of BEIIb activity and/or protein in rice, low activity-type SSIIa in japonica rice was associated with the starch granules (Fig. 3C). However, when the α-helix derived from the sixth exon in SSIIa is absent, it was no longer able to associate with the starch granules even in the absence of BEIIb activity. This result points to potential alterations in protein-protein and protein-glucan interaction in ss2a be2b (+).

Fig. 2. Native-PAGE activity staining of starch biosynthetic enzymes from crude extracts of developing seeds. (A) Starch synthase (SS) activity staining in the presence of ADP-glucose as a substrate. The acrylamide gel contains 0.05 % maize amylopectin as a primer. SSI, SSIIa, and SSIIIa activity bands are marked by horizontal arrowheads. (B) Branching enzyme (BE) activity staining using 7.5 % acrylamide gel containing 0.0001 % glycogen. BEI, BEIIa, BEIIb, and Pho1 activity bands are marked by horizontal arrowheads. (C) SS activity staining in the presence of ADP-glucose and 0.8 % oyster glycogen. SSIIIa and SSI activity bands are marked by horizontal arrowheads. (D) Debranching enzyme activity staining using 5 % acrylamide gel containing 0.4 % potato amylopectin. ISA, PUL, and Pho1 are marked by horizontal arrowheads. Panels A, C, and D were prepared from a single gel. The vertical black lines indicate the position in which the gel was connected.
Molecular mass distribution of starch biosynthetic enzyme complexes in ss2a be2b (+) double mutant.

To determine how the absence of SSIIa and BEIib activities affects the formation of protein complexes, soluble proteins were separated by gel filtration chromatography. The eluted fractions were analyzed by native-PAGE activity staining (Fig. S2; see J. Appl. Glycosci. Web site) and denatured samples were evaluated by Western blotting (Fig. 4).

In the wild-type japonica rice, SSIIa was eluted in fractions 2 to 11 and strong SSIIa Western blot signals were obtained in fractions 7 to 9 (Fig. 4). However, SSIIa activity was not detected in wild-type japonica rice (Fig. S2A; see J. Appl. Glycosci. Web site) because SSIIa in japonica rice has lower SSIIa activity than that of indica rice (Fig. S2A; see J. Appl. Glycosci. Web site).16) Although SSIIa protein was hardly detected in the SP fraction of ss2a single mutant (Fig. 3A), small amounts of truncated SSIIa protein were detected after gel filtration chromatography in fractions 3 and 4 in ss2a (Fig. 4). A possible explanation for this observation is that proteins were more concentrated via gel filtration chromatography procedure. The elution pattern of SSIIa in be2b (+) single mutant resembled that of the wild-type although the amounts of SSIIa in fractions 1 to 3 and 8 in be2b (+) were greater (Fig. 4). Surprisingly, the elution pattern of truncated SSIIa in ss2a be2b (+) differed from that of the wild-type japonica rice and be2b (+) single mutant. Most of the truncated SSIIa protein eluted in fractions 2 and 3 in ss2a be2b (+), which eluted in higher molecular mass fractions than those of ss2a single mutant (Fig. 4). As expected, SSIIa activity was not detected in ss2a be2b (+) (Fig. S2C; see J. Appl. Glycosci. Web site). These results suggest that the truncated inactive SSIIa in ss2a and ss2a be2b (+) may aggregate with itself or associate with other starch biosynthetic enzymes; therefore, protecting it from degradation (Fig. 4).

BEIib eluted in fractions 1 to 12 and BEIib Western blot signals were higher in fractions 11 to 12 of wild-type japonica rice (Fig. 4). By contrast, the elution pattern of BEIib in ss2a be2b (+) was similar to that of the be2b (+) single mutant but different from that of the wild-type in that a large proportion of BEIib protein eluted in fractions 2 and 3 (Fig. 4). BEIib proteins in fractions 2 and 3 of ss2a be2b (+) were inactive (Fig. S2D; see J. Appl. Glycosci. Web site).

The elution patterns of SSIIa were similar among all four lines, and SSIIa eluted predominantly in fractions 2 and 3 in the > 700 kDa high molecular mass fractions (Figs. 4 and S2A; see J. Appl. Glycosci. Web site).17) The elution patterns of other starch biosynthetic proteins, such as SSI, SSIVb, BEI, BEIib, and Pho1, in ss2a be2b (+) were altered to some degree. SSI in wild-type japonica rice and ss2a single mutant eluted over a wide range (i.e., fractions 3 to 12) of molecular mass (Fig. 4).16) SSI activity bands were also detected in fractions 3 to 12.16) The elution pattern of SSI in be2b (+) single mutant was different from that of ss2a be2b (+). In be2b (+) single mutant, SSI was broadly eluted and most abundant in fractions 2 and 8 to 11.17) By contrast, SSI protein amounts and its activity in ss2a be2b (+) were detected primarily in fractions 9 to 11 (Figs. 4 and S2A, B, C; see J. Appl. Glycosci. Web site).

SSIVb in ss2a be2b (+) eluted in a wider range of molecular mass fractions (Fig. 4) than the wild-type and parental single mutant lines.16)17) SSIVb in ss2a be2b (+) was enriched in fractions 2 and 8, suggesting that SSIVb eluted in two peaks, namely, at the > 700 kDa high molecular mass fraction and the 150 to 300 kDa lower molecular mass fraction (Fig. 4). The second peak of SSIVb at 150 to 300 kDa was unique to ss2a be2b (+) and was not seen in the wild-type and parental single mutant lines.16)17) BEI in the wild-type japonica rice eluted in fractions 3 to 12, which represents a wide range of molecular mass complexes (Fig. 4). A larger amount of BEI was detected in fractions 11 to 12 as shown by Western blotting (Fig. 4).17) The strength of BEI activity correlated with the amount of BEI protein and its activity was highest in the < 150 kDa low molecular mass fractions.16) The amounts of BEI proteins > 300 kDa in parental single mutant lines were greater the wild-type (Fig. 4).16)17) On the other hand, BEI in ss2a be2b (+) was enriched in fractions 9 to 12, which corresponded to higher activities (Figs. 4 and S2D, E; see J. Appl. Glycosci. Web site).

BEIib in the wild-type japonica rice and ss2a single mutant was more abundant in fractions 8 to 12 (Fig. 4).17) BEIib activity followed similar trends to its abundance as
shown by Western blotting. On the other hand, BEIIa in ss2a be2b (+) was concentrated in fractions 8 to 10 (Fig. 4) and its activity was also highest in those fractions (Fig. S2D; see J. Appl. Glycosci. Web site). The elution pattern of BEIIa in ss2a be2b (+) was different from that in be2b (+) single mutant, where a considerable amount of BEIIa eluted in fractions 2 to 10 (Fig. 4).

ISA1 and PUL activities in the wild-type and ss2a be2b (+) were similar and found in a wide range of molecular mass (Figs. S2F and S3; see J. Appl. Glycosci. Web site). The elution patterns of Pho1 in the wild-type and ss2a be2b (+) were also similar. Pho1 eluted in fractions 5 to 10 in the wild-type and in fractions 5 to 8 in ss2a be2b (+) (Fig. 4) and Pho1 activity was also found in the same fractions (Figs. S2D, S2F, and S3; see J. Appl. Glycosci. Web site).

Identification of SSIIa and BEIIb proteins eluted in the high molecular mass fraction of gel filtration chromatography.

Western blotting of fractions obtained from the gel filtration chromatography revealed that large proportions of truncated inactive SSIIa and inactive BEIIb in ss2a be2b (+) were in the high molecular mass fractions (i.e., fractions 2 and 3), unlike in wild-type japonica rice (Fig. 4). In addition, SSIIa proteins were hardly detected in the SP fraction but were present in small amounts in LBP fraction of ss2a single mutant (Figs. 3A and B). To determine whether the truncated inactive SSIIa and inactive BEIIb were present in the high molecular mass fractions in ss2a be2b (+), they were subjected to LC-MS/MS analyses. Fractions 2 and 3 from gel filtration chromatography were pooled and analyzed by SDS-PAGE. This was followed by Western blotting of identical gels (Fig. 5A). SDS-PAGE gel was stained with Coomassie Brilliant Blue. After staining, the gel region corresponding to molecular mass of 80 to 90 kDa in ss2a be2b (+) was excised and divided into five pieces, and labeled as a, b, c, d, and e. The rationale for selecting this gel region is based on Western blot signals of SSIIa since the predicted molecular mass of SSIIa and BEIIb is about 87 kDa (Fig. 5A). The excised gel slices were
subjected to trypsin digestion followed by LC-MS/MS to determine the identity and composition of the separated proteins.

Amino acid sequences of SSIIa (UniProtKB accession number: Q0DDDE3) and BEIIb (UniProtKB accession number: BAA03738) are shown in Figs. 5B and C with the peptide fragments identified by LC-MS/MS highlighted in gray. SSIIa was most abundant in the gel slice designated as ‘c’ (Figs. 5A and D), while BEIIb was found mainly in the gel slice designated as ‘c’ based on these gel fragments receiving the highest probability scores (Figs. 5A and D). Pyruvate orthophosphate dikinase (PPDK) was enriched in gel slice ‘c’ and a small amount of BEI was detected in gel slices ‘d’ and ‘e’ (Figs. 5A and D). The BEIIb peptide fragments identified by LC-MS/MS in ss2a be2b (+) revealed a Gly644Arg substitution (Fig. 5C). This finding is consistent with the expected amino acid sequence from the nucleotide substitution of BEIIb in the parental be2b (+) mutant. Unfortunately, LC-MS/MS could not identify the peptide fragment of truncated SSIIa in ss2a be2b (+), where exon 6 was missing and exons 5 and 7 merged. The inability to identify truncated SSIIa in ss2a be2b (+) is likely due to peptide fragments that frequently contain arginine and lysine. These amino acids are typically digested by trypsin (Fig. 5B).

**Interaction of starch biosynthetic enzymes analyzed by co-immunoprecipitation in ss2a be2b (+) double mutant.**

To determine whether truncated inactive SSIIa and inactive BEIIb associated with other starch biosynthetic enzymes, co-immunoprecipitation was performed on soluble proteins extracted from developing seed using antisera against starch biosynthetic proteins. Co-immunoprecipitated proteins were then probed with isozyme-specific antibodies (Fig. 6). Inactive BEIIb in ss2a be2b (+) associated with SSIIa, SSIIa, SSIIa, SSIVb, BEI, and BEIIa. BEIIa associated with Pho1, but inactive BEIIb did not in ss2a be2b (+) (Fig. 6). In ss2a be2b (+), pairwise interactions between SSIIa and BEIIb, SSIIa and SSIVb, SSIVb and BEIIa, and BEI and BEIIb were observed. Furthermore, interactions between SSIIa and SSIIa, SSIIa and SSIIa, SSIIa and BEIIb, SSIIa and BEIIa, and SSIIa and SSIIa were detected, but these interactions were not reciprocal (first acronym, antibody used for immunoprecipitation; second acronym, isozyome detected by western blotting) (Fig. 6). Interactions between the proteins were confirmed in at least one out of three replicates. The interaction of inactive BEIIb with multiple starch biosynthetic enzymes was also observed in be2b (+) single mutant (Fig. 5C; see J. Appl. Glycosci. Web site). The number of interactions between BEIIb and other starch biosynthetic enzymes was also observed in be2b (+) single mutant was lower than that in ss2a single mutant and ss2a be2b (+) (Figs. 6 and S4; see J. Appl. Glycosci. Web site). In addition, the number of interactions between SSIIa and other starch biosynthetic enzymes in the wild-type and be2b (+) single mutant were greater than that in ss2a single mutant and ss2a be2b (+) (Figs. 6 and S4; see J. Appl. Glycosci. Web site). These results suggest that the interacting partner of SSIIa may be altered because of mutations in both SSIIa and BEIIb in ss2a be2b (+).

**Predicted protein complexes in ss2a be2b (+).**

Complexes of starch biosynthetic enzymes were predicted based on the results of gel filtration chromatography, LC-MS/MS identification of proteins eluted in the high molecular mass fractions (i.e., fractions 2 and 3), and co-immunoprecipitation studies. The proposed interactions among starch biosynthetic enzymes in the wild-type (Fig. 7A), ss2a (Fig. 7B), be2b (+) (Fig. 7C), and ss2a be2b (+) (Fig. 7D) are shown schematically.

Gel filtration chromatography of wild-type, Kinmaze, showed co-elution of SSI, SSIIa, SSIVb, BEI, BEIIa, and BEIIb over broad molecular mass ranges (Fig. 4). Interaction of SSIIa or SSIVb with other starch biosynthetic enzymes were observed by co-immunoprecipitation, and interaction between SSI and SSIIa was notable (Fig. S4A; see J. Appl. Glycosci. Web site). These support that the formation of SSI-SSIIa-BEIIa trimeric protein complexes as well as multi-protein high molecular mass complexes in wild-type (Fig. 7A).

In contrast, small amount of truncated inactive SSIIa were eluted only in the high molecular mass fractions (Fr 3 and 4) in ss2a single mutant (Fig. 4). Absence of SSIIa from the SSI-SSIIa-BEIIa trimeric protein complex was initially thought to be compensated by SSI. However, the present co-immunoprecipitation study showed weaker or less interactions between SSI and BEIIb as well as among other starch biosynthetic enzymes except for SSIVb (Fig. 5B; see J. Appl. Glycosci. Web site). These suggest absence of SSI-SSIIa-BEIIa trimeric protein complex in ss2a single mutant. Instead, SSIVb may form alternative protein complexes with SSI, BEI, BEIIa, and/or BEIIb in ss2a single mutant (Fig. 7B). When the major SS isozymes were simultaneously reduced in ss1 L ss3a, small amount of truncated inactive SSIIa were detected, but truncated inactive SSIIa may not be eluted in the high molecular mass fractions (i.e., fractions 2 and 3). The amount of truncated inactive SSIIa eluted in these fractions were more than that of ss2a single mutant (Fig. 7C). The elution profiles of gel filtration chromatography in be2b (+) single mutant were surprisingly different from wild-type, and almost all starch biosynthetic proteins except for Pho1 were co-eluted in high molecular mass fractions > 700 kDa (Fig. 4). As observed by co-immunoprecipitation, inactive BEIIb was associated with almost all starch biosynthetic enzymes (Fig. 4C; see J. Appl. Glycosci. Web site). These suggest that inactive BEIIb recruited starch biosynthetic enzymes to form multi-protein high molecular mass protein complexes (Fig. 7C). Co-immunoprecipitation study also suggested that BEIIa likely replaced with inactive BEIIb to form SSI-SSIIa-SSIIa trimeric complex in be2b (+) single mutant (Fig. 7C).

The elution profiles of gel filtration chromatography in ss2a be2b (+) was similar to be2b (+), and the truncated inactive SSIIa in ss2a be2b (+) was co-eluted with inactive BEIIb in fractions 2 and 3. The amount of truncated inactive SSIIa eluted in these fractions were more than that of ss2a single mutant (Fig. 4). These suggest that inactive BEIIb recruited the truncated inactive SSIIa to high molecular mass complexes in ss2a be2b (+) and prevented truncated inactive SSIIa from degradation by protease (Fig. 7D). Amount of SSIVb in < 300 kDa was greater than that of the wild-type, ss2a, and be2b (+). This suggests that SSIVb formed alternative
Identification of SSIIa and BEIIb in ss2a be2b (+).

(A) Fractions 2 and 3 (Fig. 4), which correspond to high molecular mass fractions, were pooled and subjected to SDS-PAGE (Left panel). The gel piece adjacent to the molecular markers 80–90 kDa was excised into five pieces and designated a–e as shown. This region of the gel corresponds to the migration position of SSIIa and BEIIb, which have a predicted molecular mass of 87 kDa. Excised gel pieces were digested by trypsin and analyzed by LC-MS/MS. Peptide fragments were identified using MASCOT search engine against Oryza Sativa Japonica database. Western blotting of an identical gel used for the identification of SSIIa (Right panel).

(B) Amino acid sequence of wild-type SSIIa (accession number: Q0DDE3). Gray highlights the peptides identified by LC-MS/MS. The bold font indicates peptides that were potentially deleted in ss2a due to a mutation in the sixth exon.

(C) Amino acid sequence of wild-type BEIIb (accession number: BAA03738). Gray highlights peptides identified by LC-MS/MS. The glycine residue of wild-type BEIIb indicated in bold was mutated to an arginine residue in be2b (+).

(D) List of identified proteins corresponding to the gel slices in (A). The probability score was calculated as -10*LOG10(P), where P is the absolute probability.

Fig. 5. Identification of SSIIa and BEIIb in ss2a be2b (+).
protein complexes with BEIIa, and possibly with SSI, BEI or be2b (+) in ss2a be2b (+) (Fig. 6). In addition to known starch biosynthetic enzymes, other enzymes such as PPDK (Fig. 5) and unknown protein may participate in the formation of protein complexes in ss2a be2b (+) (Fig. 7D).

BEIib in the be2b (+) background has a Gly644Arg mutation leading to an inactive enzyme. It remains to be determined whether this Gly644Arg mutation in BEIib alters protein-protein interactions or recruits other starch biosynthetic proteins to form high molecular mass protein complexes. Alternatively, the Gly644Arg mutation in BEIib could facilitate the association among starch biosynthetic proteins through interactions with glucans.

The differences between the elution pattern of be2b (+) and ss2a be2b (+) could explain the role of SSIIa, here japonica-type SSIIa, in the formation of protein complexes in be2b (+). In be2b (+) single mutant, SSIIa proteins co-eluted with SSIVb, BEI, BEIIa and inactive BEIib in a wide range of molecular mass fractions, namely, fractions 2 to 12 (Fig. 4). This observation suggests that multiple combinations of complexes among these enzymes could form in be2b (+). By contrast, truncated inactive SSIIa was not detected in fractions 5 to 12 in ss2a be2b (+) (Fig. 4), and only small amounts of SSII, BEI, and BEIIa in ss2a be2b (+) were detected in fractions 2 to 7. SSII, BEI, and BEIIa were mostly present in fractions 9 to 11, which corresponded to protein complexes that were < 300 kDa (Fig. 4). These findings indicate that SSII, BEI, and BEIIa were not able to form protein complexes that are > 300 kDa in the absence of SSIIa (Fig. 4). Therefore, SSIIa in be2b (+) single mutant was responsible for the formation of protein complexes with SSII, BEI, BEIIa and inactive BEIib in the > 300 kDa molecular mass fraction. These findings reinforce the importance of SSIIa in protein complex formation since SSIIa is known to be present at the core of the SSIIa-BEIib protein complex in developing endosperm of maize.15

In addition to truncated inactive SSIIa and inactive BEIib, proteins involved in the formation of high molecular mass protein complexes included SSIIIa, SSIVb, BEI, Pho1, and PPDK (Figs. 4, 5, and 6). The interaction between PPDK with other starch biosynthetic enzymes such as SSIIa and ADP-glucose pyrophosphorylase is proposed to bridge associations of this enzyme complex with SSIIa and BEIib in maize.29 The findings made in maize support the possibility that PPDK is also a component of protein complexes in rice. Although the exact mechanisms by which PPDK modulates starch structure remains unknown, it was shown that the absence of PPDK leads to floury rice endosperms with altered amylpectin branch structure. The altered amylpectin branch structure in the absence of PPDK supports the involvement of this enzyme in starch biosynthesis.29 The significance of the formation of high molecular mass protein complexes for starch biosynthesis remains uncertain. However, accumulating evidence suggests that these high molecular mass protein complexes (i.e., > 700 kDa) may facilitate the formation of smaller (i.e., 150–300 kDa) functional protein complexes as suggested in maize.15

The structure and physicochemical properties of starch in ss2a be2b (+) were closely resembled that of the be2b (+) single mutant.19 Amounts of short amylpectin chains were reduced but those of long amylpectin chains were increased relative to the wild-type.18 This led to an increase in gelatinization temperature in ss2a be2b (+).30 Apparent amylase content, starch content and seed weight were higher in the ss2a be2b (+) than those of the parental be2b (+) single mutant.19 These suggest that formation of alternative starch biosynthetic protein complexes allowed to compensate the absence of two major enzymes and efficiently synthesized starch rather than controlling the structure of amylpectin.

**CONCLUSION**

The present study revealed that SSII-SSIIa-BEIib trimeric protein complex found in wild-type was absent in ss2a be2b (+). In addition, inactive BEIib protein in ss2a be2b (+) double mutant formed high molecular mass protein complexes in the soluble fraction of developing rice endosperm, which was similar to observations made in be2b (+) single mutant.17 In ss2a be2b (+) double mutant, large amounts of truncated inactive SSIIa were also present in the high molecular mass fractions together with SSIIIa, SSIVb, PPDK and inactive BEIib. By contrast, ss2a single mutant had less SSIIa than ss2a be2b (+) double mutant in the high molecular mass fractions. These results suggest that in ss2a be2b (+), truncated inactive SSIIa may be protected from protease degradation by associating with inactive BEIib and other starch biosynthetic enzymes.

In ss2a be2b (+), SSII, BEI, and BEIIa eluted in the < 300 kDa molecular mass fractions. By contrast, SSII, BEI, and BEIIa exhibited a broader range of elution patterns in be2b (+) single mutant. In addition to being detected in the < 300 kDa molecular mass fractions, these enzymes eluted in the > 300 kDa molecular mass fractions in be2b (+) single mutant. These results suggest that the SSIIa protein is responsible for interactions among SSII, BEI, BEIIa, and inactive BEIib in the be2b (+) mutant. These results further indicate that SSII, BEI, and BEIIa may form alternative protein complexes in the absence of SSIIa and BEIib. Interactions between inactive BEIib and multiple starch biosynthetic enzymes
were also observed. Future analyses of be2b and ss2a single mutants bearing different mutation sites as well as other starch biosynthetic enzyme double mutant combinations could lead to a more detailed understanding of protein complex formation in cereal endosperm.

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENTS

This study was supported in part by the Science and Technology Research Promotion Program for Agriculture, Forestry and Fisheries and Food Industry [25033AB and 28029C to N.F.]; the President’s Funds of Akita Prefectural University [N.F. and N.C.]; Grant-in-Aid for the JSPS fellows from Japan Society for the Promotion of Science [#15J40176, JP18J40020, 20K05961 to N.C., and 19H01608 to N.F.]; and the Japan Society for the Promotion of Science [#16K18571 and JP18K14438 to N.C.]. The authors thank Japan Proteomics Co., LTD. for LC-MS/MS analysis. The authors also thank Yuko Nakaizumi in Akita Prefectural University for growing rice plants.

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