The Starch-Debranching Enzymes Isoamylase and Pullulanase Are Both Involved in Amylopectin Biosynthesis in Rice Endosperm[1]

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The activities of the two types of starch debranching enzymes, isoamylase and pullulanase, were greatly reduced in endosperms of allelic *sugary-1* mutants of rice (*Oryza sativa*), with the decrease more pronounced for isoamylase than for pullulanase. However, the decrease in isoamylase activity was not related to the magnitude of the *sugary* phenotype (the proportion of the phytoglycogen region of the endosperm), as observed with pullulanase. In the moderately mutated line EM-5, the pullulanase activity was markedly lower in the phytoglycogen region than in the starch region, and isoamylase activity was extremely low or completely lost in the whole endosperm tissue. These results suggest that both debranching enzymes are involved in amylopectin biosynthesis in rice endosperm. We presume that isoamylase plays a predominant role in amylopectin synthesis, but pullulanase is also essential or can compensate for the role of isoamylase in the construction of the amylopectin multiple-cluster structure. It is highly possible that isoamylase was modified in some *sugary-1* mutants such as EM-273 and EM-5, since it was present in significant and trace amounts, respectively, in these mutants but was apparently inactive. The results show that the *Sugary-1* gene encodes the isoamylase gene of the rice genome.

The super-cluster structure of amylopectin (Gallant et al., 1997) might have evolved as a fitting strategy for plant survival and must be accomplished by well-refined regulation of a network of numerous enzyme actions. The fine structure of amylopectin is distinct from that of glycogen in animals and bacteria in that glycogen is randomly branched, the branches are more numerous, and the chains are shorter compared with amylopectin. Biochemical analyses of *sugary*-type mutants of maize, rice (*Oryza sativa*), Arabidopsis, and *Chlamydomonas reinhardtii* have provided new insights needed to understand the mechanism of synthesis and construction of the amylopectin fine structure (Ball et al., 1996; Nakamura, 1996; Smith et al., 1997). Since these mutants accumulate phytoglycogen with more branches than amylopectin (Shannon and Garwood, 1984; Nakamura, 1996), analysis of the biochemical lesion in these mutants can give clues for the assessment of the functions of enzymes involved in amylopectin biosynthesis.

Pan and Nelson (1984) first reported that the *sugary-1* phenotype is caused by the loss of the activity of a pullulanase-type starch debranching enzyme, suggesting that the debranching enzyme is also involved in starch biosynthesis. That report sharply contrasted with the well-accepted idea that the starch debranching enzymes (pullulanase and isoamylase) are only involved in starch degradation in conjunction with other hydrolytic activities. James et al. (1995) later showed by transposon tagging that the *Sugary-1* gene of maize encodes an isoamylase-like enzyme. Recently, their group reported that the *Sugary-1* gene product possesses isoamylase activity, and that *sugary-1* mutants are deficient in both isoamylase and pullulanase (Rahman et al., 1998; Beatty et al., 1999).

Nakamura et al. (1992, 1996b) assayed the major enzymes of starch and Suc metabolism in developing endosperms of *sugary-1* rice mutants. They found that while both pullulanase and starch branching enzyme I activities were significantly reduced in the mutants, the reduction of pullulanase activity was much more pronounced than that of starch branching enzyme I activity; therefore, the ratio of pullulanase to starch branching enzyme I activity is markedly lower in *sugary-1* endosperm than in normal wild-type endosperm (Nakamura et al., 1996b). In other studies, it was observed that endosperms of four out of five allelic *sugary-1* rice mutants were differentiated into an iodine-staining, starch-containing region and an iodine-non-staining, phytoglycogen-containing region (Nakamura et al., 1997). Moreover, in *sugary-1* endosperm tissues with a more extensive phytoglycogen region, the amount of phytoglycogen in the total α-polyglucan content and the reduction in the pullulanase activity become greater (Nakamura et al., 1997).

Mouille et al. (1996) isolated in *C. reinhardtii sugary*-like *sta7* mutants in which starch is replaced by a small amount of phytoglycogen. Isoamylase is lacking while pullulanase activity is unaffected in these mutants.

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Zeeman et al. (1998) reported an Arabidopsis mutant having both starch and phytoglycogen in leaf chloroplasts. The mutant lacks isoamylase, but the levels of pullulanase, starch branching enzyme, and starch synthase are normal.

The above observations indicate that both isoamylase and pullulanase are involved in the determination of amylopectin structure, and that the loss or reduction in these debranching enzymes results in phytoglycogen production at the expense of amylopectin synthesis. However, whether both types of starch debranching enzymes play distinct roles or if both enzymes are essential for the construction of amylopectin fine structure still remains to be elucidated. More detailed data are needed to understand how debranching enzymes, together with starch branching enzymes and starch synthase, are involved in determining amylopectin structure.

The present investigation has been carried out to examine the relationship between the activities of starch debranching enzymes and the alteration of amylopectin structure in various *sugary-1* endosperms of rice by partitioning the endosperm into the starch region and the phytoglycogen region. In this paper, changes in isoamylase activities due to *sugary-1* mutations were determined by the native-PAGE/activity staining method, which allows the detection of isoamylase activity in the presence of other amylolytic enzymes. The present investigation provides strong evidence supporting the involvement of both isoamylase and pullulanase in amylopectin biosynthesis in rice endosperm. Structural features of polyglucans in the starch and phytoglycogen regions of various *sugary-1* mutant endosperms have also been analyzed in detail to determine whether polyglucan structure changes consecutively or in a step-wise manner in accordance with activity changes in debranching enzymes and other enzymes, and whether there are polyglucan structures common to the starch and phytoglycogen regions in every mutant line. Findings could provide important clues as to what factors influence the dramatic polyglucan structural changes between amylopectin and phytoglycogen. Structural differences between wild-type amylopectin and amylopectin-like glucan in the starch region of *sugary-1* endosperm are also discussed.

MATERIALS AND METHODS

Plant Materials

In this study, five allelic lines of *sugary-1* mutants (EM-5, EM-41, EM-273, EM-914, and EM-935) of rice (*Oryza sativa*) derived from two parent cvs (cv Kinmaze for the former three lines and cv Taichung 65 for the latter two lines) were used (Nakamura et al., 1997). The *sugary-1* mutants were induced by treatment with 1.0 mM *N*-methyl-*N*-nitrosourea (Satoh and Omura, 1979). All mutant lines had been maintained by repeated self-pollination for several years. Rice plants were grown during the summer months under natural environmental conditions in an experimental field of the National Institute of Agrobiological Resources (Ibaraki, Japan). As an enzyme source, the ripening grains at the fully expanded milking stage (the late-milking stage) were collected and stored at −80°C until use. For preparation of starch samples, mature grains were harvested before complete dryness and stored at −20°C.

Preparation of Kernel Cross-Sections for Light and Electron Microscopy

Rice kernel samples taken at the late-milking stage were used. The cross-sections were prepared from the middle part of the kernels, stained with I_2/KI solution as described previously (Nakamura et al., 1997), and observed under a light microscope.

For scanning electron microscopic observations, whole rice kernels at the late-milking to dough stage were rapidly frozen in slush liquid nitrogen (−210°C) and then freeze-dried under vacuum for 20 h at −60°C in a high-vacuum freeze-dryer (10^{-3} Pa, model OTD-5SF, Oka Science, Tokyo). The dried specimens were sectioned transversely and then affixed onto brass stubs, sputter-coated with gold, and examined with a scanning electron microscope (10 kV, model T300, JEOL, Tokyo).

Separation of *sugary-1* Mutant Endosperm into the Starch Region and the Phytoglycogen Region

Grains from EM-5 at the late-milking stage or at the mature stage before complete dryness were dehulled, and 1-mm sections across the short axis were taken at 0°C to 4°C with a razor blade (Fig. 1). Both sides of the excised disc, including the starch region, were removed and the remaining middle part was then divided in half. Each of the halves was cut with a razor blade into four sections of the same width (Fig. 1B). Each section was used for enzyme assay or for the analysis of polyglucan structure by high-performance anion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD).

Analysis of α-Polyglucan Structure

Determination of the Distribution of α-1,4-Glucan Chain Length of α-Polysaccharides by HPAEC-PAD

Ten average-size mature grains before complete dryness were dehulled and their embryo and pericarp were removed. They were then suspended in 5 mL of methanol, boiled for 10 min, and the homogenate was centrifuged at 2,500g for 10 min. The precipitated polyglucan fraction was washed twice with 1 mL of 90% (v/v) methanol, suspended in 5 mL of distilled water, and then boiled for 60 min. The gelatinized polyglucan sample was added to 50 μL of 600 mM sodium acetate buffer (pH 4.4) and 10 μL of 2% (w/v) NaN_3, and hydrolyzed by adding 10 μL of *Pseudomonas amyloderamosa* isoamylase (1,400 units, Seikagaku, Tokyo) at 37°C for 24 h. The hydroxyl groups of the debranched glucans were reduced with 25 mg of sodium borohydride under alkaline pH conditions for 20 h by the method of Nagamine and Komae (1996). The precipitate was dried in vacuo at room temperature. The reduced isoamylolysate sample was dissolved in 30 μL of 1 M NaOH for 60 min and diluted with 270 μL of distilled
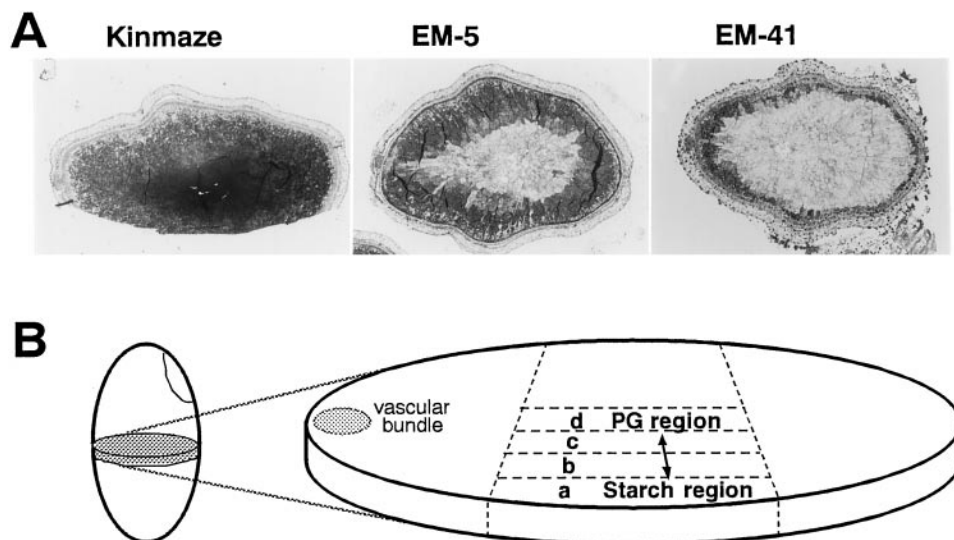
Figure 1. A, Light micrographs of cross-sections through kernels at the late-milking stage of rice *sugary-1* mutants EM-5 and EM-41 and their parent cv Kinmaze. Sections were stained with iodine solution. B, Schematic representation for partitioning the phytoglycogen (PG) region and the starch region of *sugary* endosperm.

water. A 50-μL aliquot of the preparation was injected into a BioLC (model DX-500, Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector and a CarboPac PA-1 column (4 mm × 25 cm). Size fractionation of α-1,4-glucans was performed with a linear gradient of sodium acetate (50–500 mM) in 0.1 M NaOH at a flow rate of 1 mL min^{-1}.

Molecular Size Separation of α-Polyglucans by Sephacryl S-1000 Chromatography

Two hundred endosperms were sectioned as shown in Figure 1. Combined samples for each section were separately ground in a mortar and pestle, and suspended with 4 mL of 1 M NaOH. After 30 min at room temperature, 4 mL of distilled water was added to the sample suspension and then centrifuged at 2,000g for 5 min at 25°C. Four milliliters of the supernatant was applied onto a Sephacryl S-1000 (Pharmacia Biotech, Uppsala) column (2.0 cm diameter; 60 cm length) that had been equilibrated with the 0.1 M NaOH, 0.2% NaCl solution at a flow rate of about 18.3 mL h^{-1} at room temperature. Fractions were taken at 200-drop intervals (equivalent to 6.6 mL).

The total carbohydrate content was measured by the phenolic sulfuric method of Duvois et al. (1956). The absorbance of the phenolic sulfuric mixture was measured at 490 nm. For determination of λ_{max}, the absorbance of the polysaccharide-I$_2$ complex was obtained in the 450 to 700 nm range. Both procedures were described in detail by Nakamura et al. (1997).

Enzyme Activity Measurements in the Outer and Inner Regions of the Endosperm

For assay of isoamylase activities, the outer region (a + b in Fig. 1B) and the inner region (c + d) of the seed endosperm at the late-milking stage of cv Kinmaze, EM-5, and EM-41 were prepared from 30 kernels in 0.2 mL of a grinding buffer solution containing 50 mM imidazole-HCl (pH 7.4), 8 mM MgCl$_2$, 12.5% (v/v) glycerol, and 50 mM 2-mercaptoethanol. Extracts were centrifuged at 15,000g for 5 min at 4°C, and supernatants were removed and kept on ice before use.

For assay of other enzymes, enzyme extracts from the outer and inner regions were prepared from 10 developing kernels in 0.2 mL of the same buffer solution as described above. For measurement of starch branching enzyme activity, the extracts were precipitated before use with ammonium sulfate (60% saturation) to remove phytoglycogen, which interferes with the assay (Nakamura et al., 1996b).

Partial Purification of Isoamylase from Endosperms of *sugary-1* Mutants

For zymogram detection of the activity of isoamylase from various *sugary-1* mutants (Fig. 5A), 20 kernels at the late-milking stage were extracted in a chilled-glass homogenizer in 2 mL of a grinding buffer at 4°C. The crude extracts were centrifuged at 15,000g for 5 min at 4°C and the supernatants were removed. The extract was filtered with a cellulose acetate membrane (0.45 μm, Tosoh, Tokyo), and the filtrate was loaded onto an anion-exchange column (HiTrap-Q, Pharmacia Biotech) (1 mL) that had been equilibrated with solution A (50 mM imidazole-HCl [pH 7.4], 8 mM MgCl$_2$, and 50 mM 2-mercaptoethanol) at room temperature. The column was consecutively washed with 50 mL of solution A and 50 mL of 0.3 M NaCl in solution A until no detectable protein was eluted in the last wash. Isoamylase was eluted from the column with 0.4 M NaCl in solution A at 3 mL min^{-1}. The peak fraction (about 10 mL) of isoamylase activity was concentrated by ultrafiltration columns (Centricon-50, Millipore, Bedford, MA) to 70 μL according to the manufacturer's instructions, and stored on ice until use.

For further measurement of the isoamylase activity by zymogram, as shown in Figure 5B, the isoamylase was partially purified by anion-exchange chromatography as described above from 10 g of grains (equivalent to about 400 grains) of cv Kinmaze, EM-5, and EM-41, and precipitated with ammonium sulfate (42.5% saturation). The precipitate was redissolved in 1 mL of solution A, and then applied onto a gel-filtration column (7.8 mm × 30 cm, TSKgel G4000SW$_{XL}$, Tosoh) equilibrated with solution A. The isoamylase was eluted with solution A at a flow rate of 1 mL min^{-1} at room temperature, and the peak fraction was concentrated to 40 μL with ultrafiltration columns and stored on ice until use.

Assay of Enzymes

The activities of ADP-Glc pyrophosphorylase, starch synthase, starch branching enzyme, and pullulanase were measured at 30°C under optimal conditions for each enzyme, as described previously (Nakamura et al., 1989, 1996b). All enzymes were assayed in a range at which the velocity was proportional to the enzyme concentration and the incubation time. Each result is the mean ± SD of at least three replicate incubations.

Native-PAGE/Activity Staining of Isoamylase and Pullulanase

Native-PAGE was performed on 7.5% (w/v) acrylamide slab gels containing 0.15% (w/v) potato tuber amylopectin (Sigma, St. Louis) by modification of the methods of Davis (1964), as described previously (Yamanouchi and Nakamura, 1992). Electrophoresis was carried out at 4°C at a constant current of 15 mA. For detection of the isoamylase activity, the gel was rinsed twice and then incubated at 30°C for 2 h with 20 mL of 50 mM citric-Na$_2$HPO$_4$ (pH 6.0) containing 50 mM 2-mercaptoethanol. The isoamylase activity was visualized by staining the gel with 0.1% (w/v) I$_2$/1% (w/v) KI solution.

Protein Analysis by SDS-PAGE and Western Blotting

Proteins were separated by SDS-PAGE on a resolving gel (6 × 9 × 0.1 cm) of 10% (w/v) polyacrylamide by the method of Laemmli (1970). The proteins were transferred from the gel to a PVDF membrane (Millipore), and the blots were incubated with polyclonal antibodies raised against purified isoamylase from developing rice endosperm (Fujita et al., 1999). The immunoreactive bands were detected by the method of Towbin et al. (1979).

Localization of Pullulanase Activity in Rice Endosperm by the Red Pullulan Film Method

The dehulled rice kernel at the late-milking stage was cut into halves with a razor blade and the sectioned surface was placed on a thin 10% (w/v) polyacrylamide gel (0.5 mm thick) containing 2% or 3% (w/v) Red Pullulan (Megazyme, Warriewood, Australia) and incubated at 30°C for 30 or 60 min. After incubation, seed specimens were removed and the gel was soaked in a 25% (w/v) methanol, 7.5% (w/v) acetic acid solution. The seed region possessing pullulanase activity decolorized the area of contact on the Red Pullulan-stained gel.

Measurement of Protein Content

The protein content was determined using the protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) with a standard curve of BSA.

RESULTS

Starch and Phytoglycogen Regions of *sugary-1* Mutant Endosperms

Figure 1A shows that the endosperms of *sugary-1* rice mutants were clearly divided into the iodine-stained starch region and the non-stained phytoglycogen region, indicating that biochemical changes induced by *sugary-1* mutations brought about a dramatic alteration of polyglucan structure from amylopectin to phytoglycogen. In EM-5, the phytoglycogen region at the center part of the endosperm was significantly smaller than that in EM-41, whose starch region was restricted to a few outermost layers of the endosperm. The cross-section of the mutant kernels was divided into four segments (a–d), as shown in Figure 1B. The outermost (a) and innermost (d) sections of EM-5 endosperm were expected to be comprised predominantly if not exclusively of starch cells and phytoglycogen cells, respectively.

Distribution of Different Types of α-Polyglucans in Various *sugary-1* Mutant Endosperms

When the polyglucans from the starch region (segment a in Fig. 1B) of EM-5 endosperm were fractionated by Sephacryl S-1000 gel-filtration chromatography, it was found to be composed predominantly of amylopectin-like fractions (fraction nos. 12–15), as shown by the large peak at the λ_{max} range of 540 to 548 nm (Fig. 2, A and E). Fractions that eluted later in the chromatogram (fraction nos. 25–30) were considered to contain amylose, as judged from the higher λ_{max} value (about 610–612 nm) of the iodine-glucan. In contrast, polyglucans in the phytoglycogen region (d in Fig. 1B) was almost devoid of amylopectin-like fractions, whereas the phytoglycogen fractions (fraction nos. 20–27) increased tremendously (Fig. 2D), indicating that amylopectin was almost completely replaced by phytoglycogen in the phytoglycogen region. The smallest peak (fraction nos. 31–34) was due to free sugars such as Suc and Glc (data not shown). The intermediate sections (b and c in Fig. 1B) of EM-5 endosperm, which contained both amylopectin-like and phytoglycogen fractions, formed a sort of transition region where the phytoglycogen content increased toward the interior of the endosperm (Fig. 2, B and C).

The endosperm of EM-914, which was composed only of the phytoglycogen region, exhibited a large phytoglycogen
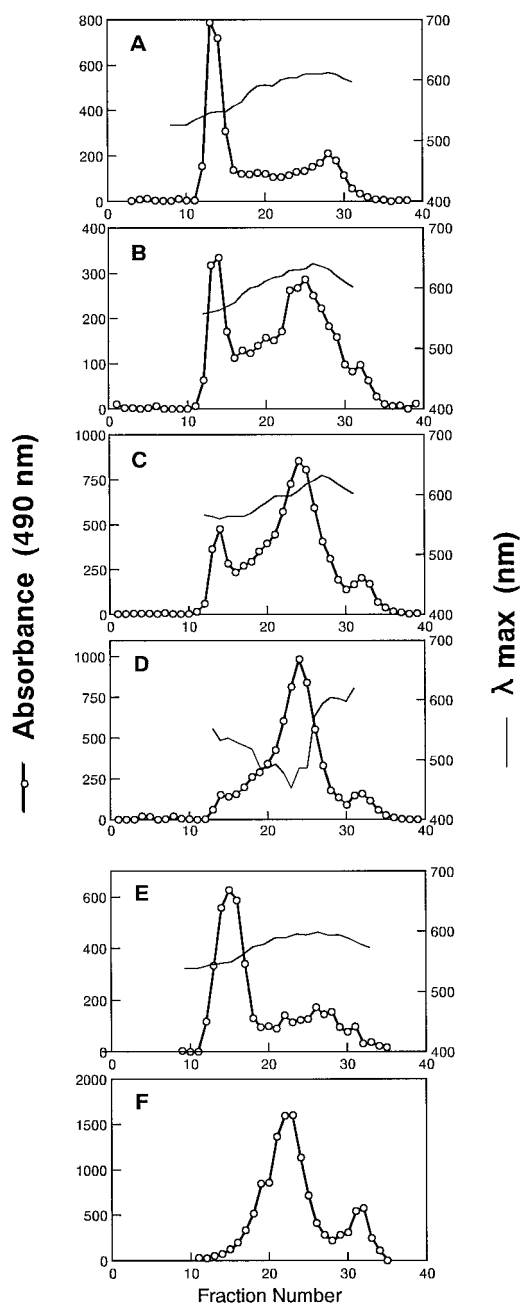
Figure 2. Size separation of polyglucans in various endosperm regions from the *sugary-1* mutants EM-5 and EM-914 by Sephacryl S-1000 gel-filtration chromatography. After chromatography, a part of each fraction was used for the measurement of carbohydrate content by the phenolic sulfuric method (O), and the other part (0.8 mL) of the fraction was added to 0.2 mL of 0.1% I_2/1% KI solution for the measurement of the λ_{max} (fine line). A, B, C, and D show results for samples prepared from sections a, b, c, and d, respectively, which were partitioned from the EM-5 endosperm as illustrated in Figure 1B. E and F show results from the whole endosperms of cv Kinmaze and EM-914, respectively.

peak without any amylopectin-like fractions, even when the sample was prepared from the whole endosperm tissue (Fig. 2F).

Chain Length Distribution of Polyglucans in Different Endosperm Regions of *sugary-1* Mutants

Chain length distribution represents the structural feature of a variety of polyglucans such as amylopectin and glycogen. Analysis by HPAEC-PAD was performed to examine the fine structures of polyglucans present in the amylopectin-like and phytoglycogen fractions of *sugary-1* mature endosperms (Fig. 3). Polyglucans in the starch region of EM-5 endosperm clearly differed in structure from the wild-type amylopectin. In the mutant, the relative amounts of B_1 ($13 \leqq DP \leqq 24$) and B_3 to B_4 chains ($DP \geqq 37$) were lower, while those in A ($DP \leqq 12$) and B_2 chains ($25 \leqq DP \leqq 36$) were higher compared with the parent cultivars (Fig. 3A) (see Hanashiro et al., 1996 for chain classification). Figure 3C shows that in EM-5, polyglucans prepared from the starch region (a in Fig. 1B) yielded an α-1,4-chain distribution pattern similar to that of the amylopectin-like fractions (fraction nos. 12–15) separated by Sephacryl S-1000 chromatography. This indicates that polyglucans in the starch region (a) of the EM-5 endosperm were accounted for by the amylopectin-like fractions eluted in the chromatogram.
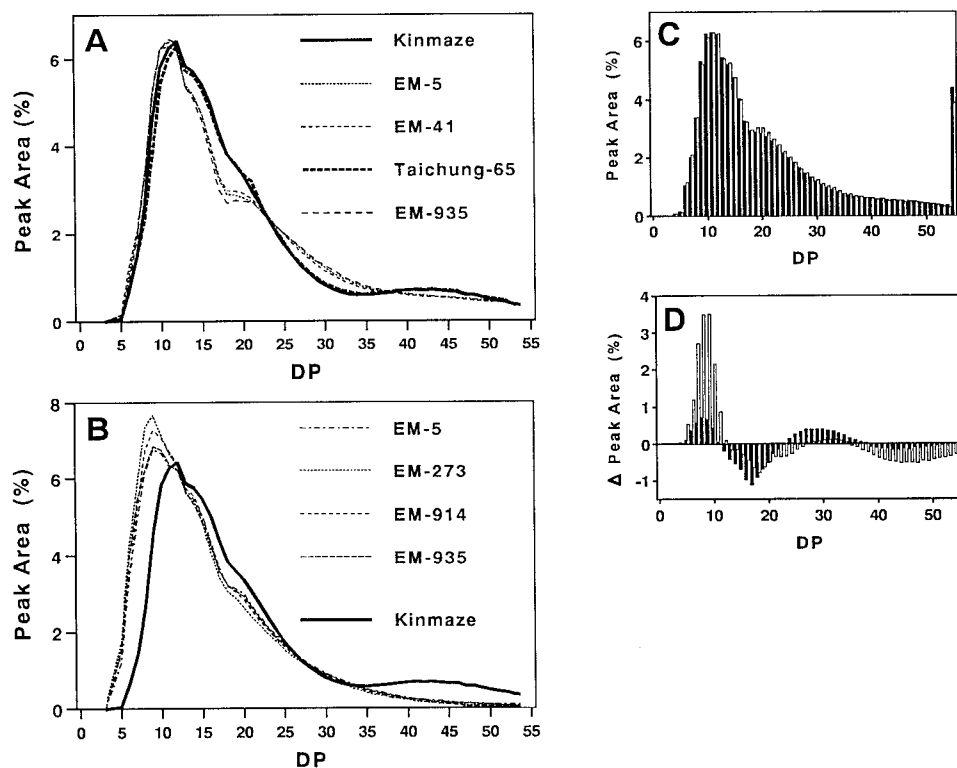
For the analysis of the chain length distribution of polyglucans in the starch regions of the EM-41 and EM-935, the amylopectin-like fractions from Sephacryl S-1000 chromatograms (fraction nos. 12–15) were injected into the HPAEC-PAD. These mutants possessed minor starch regions in their whole endosperms such that the starch region could not be divided as in Figure 1A (Nakamura et al., 1997). The chain length distribution patterns of polysaccharides in the starch regions of these mutants resembled that of EM-5 (Fig. 3A).

The fine structure of polyglucans in the phytoglycogen regions (d) of EM-5, EM-273, EM-914, and EM-935 endosperms distinctly differed from amylopectin-like structures in their starch regions (Fig. 3, A and B). These phytoglycogen structures were very similar in all mutants tested.

Figure 3D illustrates the difference in chain length distribution of polyglucans between normal amylopectin from the wild-type (cv Kinmaze) endosperm and amylopectin-like glucans or phytoglycogen in the *sugary-1* mutant EM-5. In phytoglycogen, the short chains ($5 \leqq DP \leqq 11$) dramatically increased, while the longest chains ($DP \geqq 40$) greatly decreased. In contrast, in the mutated amylopectin, chains with DP values between 12 and 21 decreased, whereas those between 24 and 36 increased. These results indicate that polyglucans in rice *sugary-1* mutant endosperm were either *sugary* amylopectin or phytoglycogen. Apparently, the mutants did not synthesize an intermediate form of polyglucan, in spite of the various biochemical changes presumably induced by the *sugary-1* mutation.

Electron Micrographs of Polyglucan Granules in *sugary-1* Mutant Endosperms

Mature rice endosperm was filled with amyloplasts packed with 3- to 5-μm polygonal granules (data not shown). Scanning electron micrograph imaging showed

Figure 3. Comparison of chain length distribution of polyglucans in the phytoglycogen and starch regions of mature endosperm of *sugary-1* mutants and their parent cultivars. Polyglucans were debranched by *P. amyloderamosa* isoamylase and reduced with sodium borohydrate. The distribution of the reduced linear glucan chains was determined by HPAEC-PAD analysis. A, Polyglucans from the starch regions of EM-5, EM-41, and EM-935 were prepared from the Sephacryl S-1000 chromatogram (fraction nos. 12–15) as shown in Figure 2. B, Polyglucans from the phytoglycogen regions of mutants EM-5, EM-273, EM-914, and EM-935 prepared as shown in Figure 1B (as d). Normal polyglucans (amylopectin) in endosperm of wild-type cv Kinmaze and cv Taichung-65 were prepared from the whole kernels without embryo (shown in A and B). C, Comparison of the results between polyglucans prepared from the starch region of EM-5 endosperm partitioned as shown in Figure 1B (as a) and those from Sephacryl S-1000 chromatogram (fraction nos. 12–15) as shown in Figure 2A. D, Difference in the distribution of chain lengths of polyglucans between wild-type amylopectin from cv Kinmaze and polyglucans from the starch region of EM-5 (black bar; data from Fig. 3A) and between wild-type amylopectin and polyglucans from the phytoglycogen region of EM-5 (white bar; data from Fig. 3B).

that the phytoglycogen region of the *sugary-1* mutants drastically changed into a reticulate structure containing fine starch granules (data not shown), which is characteristic of water-soluble polysaccharides such as glycogen. A marked change in granule morphology was also found in the starch region of EM-5. The polygonal granules remained in the amyloplasts, but numerous small granules not observed in wild-type starch crystals were also present (data not shown), indicating some changes in the fine structure of the normal amylopectin.

Effects of *sugary-1* Mutations on Activities of Enzymes Involved in Starch Synthesis in the Endosperm

Our previous reports established that pullulanase activities are specifically reduced in *sugary-1* endosperms (Nakamura et al., 1992, 1996b, 1997), and that the extent of the *sugary-1* phenotype correlates only with the decrease in pullulanase activity (Nakamura et al., 1997). There is a

need to examine whether the region-specific alteration in the polyglucan fine structure in the mutant endosperm is induced by changes in the distribution of enzymes involved in amylopectin biosynthesis. The activities of ADP-Glc pyrophosphorylase, starch synthase, starch branching enzyme, and pullulanase were quantitatively measured in both the starch and the phytoglycogen regions of EM-5 (Table I), since EM-5 was moderately phenotypically mutated and it was the only mutant where the starch and phytoglycogen regions were clearly defined (Fig. 1). Table I shows that differences in these enzyme activities between the interior and the exterior regions existed even in the wild-type endosperm. The activities of ADP-Glc pyrophosphorylase, starch branching enzyme, and starch synthase on soluble protein content basis, were basically unaffected in both endosperm regions of EM-5. In contrast, pullulanase activity was greatly reduced in the phytoglycogen region of EM-5 endosperm (Table I). Therefore, the ratio between the activities of pullulanase and starch branching enzyme was significantly lower in the phytoglycogen re-

Table I. *Activities of enzymes in rice endosperm at the late-milking stage of sugary-1 mutant EM-5 and its parent cv Kinmaze*

The enzyme extract was prepared from the outer (a+b in Fig. 1B) and the inner (c+d in Fig. 1B) regions of EM-5 and cv Kinmaze endosperms as detailed in "Materials and Methods."

cv or Line	Region	Enzyme Activity			
		ADP-Glc pyrophosphorylase	Starch synthase	Branching enzyme[a]	Pullulanase
		$nmol\ mg^{-1}\ protein\ min^{-1}$			
cv Kinmaze	Outer	84.9 ± 10.7	318.9 ± 23.9	849.4 ± 58.9	10.41 ± 0.78
	Inner	128.8 ± 26.6	340.0 ± 5.9	661.2 ± 61.2	11.79 ± 2.87
EM-5	Outer	61.6 ± 8.9	220.9 ± 19.8	617.1 ± 32.7	8.05 ± 0.19
	Inner	67.8 ± 13.4	187.1 ± 6.5	484.3 ± 11.5	2.72 ± 0.84

[a] The activity is expressed in absorbance units per mg protein.

gion, suggesting that the decrease is responsible for the structural changes in polyglucans.

Effects of *sugary-1* Mutations on the Distribution of Pullulanase

To visualize the localization of pullulanase in the endosperm, the Red Pullulan film method was used. In all *sugary-1* mutants (EM-5, EM-41, EM-273, EM-914, and EM-935) the pullulanase activity in the inner part of the endosperm was significantly lower than that in the outer part of the endosperm (Fig. 4). The starch region of the mutant endosperm had a lower pullulanase activity compared with wild-type endosperm. These results suggest that pullulanase plays an important role in expressing the *sugary-1* phenotype and, therefore, in constructing amylopectin fine structure.

Effects of *sugary-1* Mutations in the Activities and Amounts of Isoamylase in Endosperm

Because recent observations have indicated that isoamylase is involved in amylopectin biosynthesis in *sugary*-type mutants of maize endosperm (James et al., 1995; Rahman et al., 1998), *Chlamydomonas reinhardtii* (Mouille et al., 1996), and Arabidopsis leaf (Zeeman et al., 1998), the effects of *sugary-1* mutations on the isoamylase activity in rice endosperm were investigated. The rice endosperm isoamylase was partially purified using anion-exchange chromatography (Hi-Trap Q, Pharmacia Biotech) to remove other

Figure 4. Distribution of pullulanase activity in cross-section of endosperm at the late-milking stage from *sugary-1* mutants and their parent cultivars as detected by the pullulan-film method. Incubation time was 30 min and Red Pullulan content was 3%.

amylolytic enzymes such as amylases, making isoamylase activity measurable by the native-PAGE/activity staining method. Results show that isoamylase activities were dramatically reduced in the endosperms of EM-5, EM-41, EM-273, EM-914, and EM-935 relative to those in their respective wild-type parent endosperms (Fig. 5A).

Since the level of isoamylase activity in EM-41 was higher than in EM-5 even though the former was more severely mutated than the latter, the isoamylase activity was further examined using a more purified isoamylase preparation obtained with anion-exchange chromatography followed by gel-filtration chromatography. Figure 5B shows that isoamylase activity was reduced by at least one order in EM-41 compared with that in the parent cv Kinmaze, while no isoamylase activity was found in EM-5. Attempts to detect isoamylase activity in EM-5 endosperm were unsuccessful even when the endosperm was separated into inner and outer regions (Fig. 5C). No significant difference in isoamylase activity was found between the inner and the outer regions of EM-41 endosperm (Fig. 5C). All of these results strongly suggest that isoamylase plays an essential role in amylopectin biosynthesis, but the reduction in isoamylase activity is not the only biochemical factor that influences the *sugary-1* phenotype in rice endosperm.

The amount of isoamylase (83 kD) in the mutant endosperm was measured by western-blot analysis using polyclonal antibodies raised against purified isoamylase from developing rice endosperm (Fujita et al., 1999) (Fig. 6). No isoamylase bands were detected in EM-914 and EM-935, while a faint isoamylase band was found in EM-41. It is interesting to note that the isoamylase band intensity was significantly lower in EM-5 than in the wild-type cv, it was at least comparable to that in EM-41. The most remarkable result shown in Figure 6 is that the amounts of isoamylase protein in EM-273 and its parent cv Kinmaze were almost equal, notwithstanding the fact that the mutant exhibited an extremely low isoamylase activity in the endosperm (Fig. 5A). These results might indicate that the isoamylase protein was modified into an inactive form in EM-273, and possibly to an unstable and/or inactive form in EM-5. Therefore, it is reasonable to conclude that the primary event of the *sugary-1* mutation in rice is the lesion
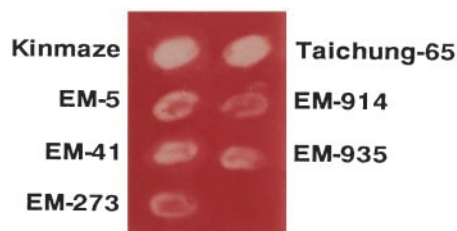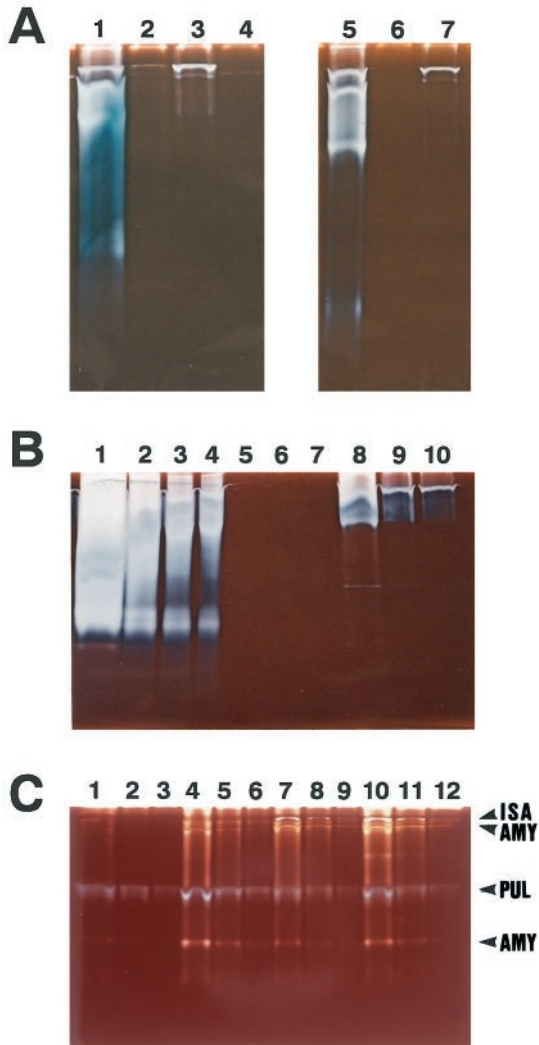
Figure 5. Activities of isoamylase in the endosperm at the late-milking stage of various *sugary-1* mutants and their parent cultivars. Isoamylase activity visualized as blue bands were detected by native-PAGE/activity staining method. A, Results with partially purified isoamylase preparations as described in "Materials and Methods." Lane 1, cv Kinmaze; lane 2, EM-5; lane 3, EM-41; lane 4, EM-273; lane 5, cv Taichung-65; lane 6, EM-914; lane 7, EM-935. Samples (5 μL) applied onto the 7.5% (w/v) polyacrylamide gel. B, Results from partially purified isoamylase samples prepared as described in "Materials and Methods" from endosperms of EM-5 and EM-41 and their parent cv Kinmaze. Volumes of samples applied onto the gel: 15 μL for lanes 1, 5, and 8; 3 μL for lanes 2, 6, and 9; 1.5 μL for lanes 3, 7, and 10; 0.75 μL for lane 4. Lanes 1 through 4, cv Kinmaze; lanes 5 through 7, EM-5; lanes 8 through 10, EM-41. C, Results from crude extracts of the inner and the outer regions of EM-5 and EM-41 endosperms fractionated as described in "Materials and Methods." Lanes 1 through 3, Inner region of EM-5 endosperm; lanes 4 through 6, outer region of EM-5 endosperm; lanes 7 through 9, inner region of EM-41 endosperm; lanes 10 through 12, outer region of EM-41 endosperm. Volumes of samples applied onto the 7.5% polyacrylamide gel: 15 μL for lanes 1, 4, 7, and 10; 5 μL for lanes 2, 5, 8, and 11; 1 μL for lanes 3, 6, 9, and 12. Arrows indicate the bands corresponding to isoamylase (ISA), pullulanase (PUL), and amylases (AMY), respectively.
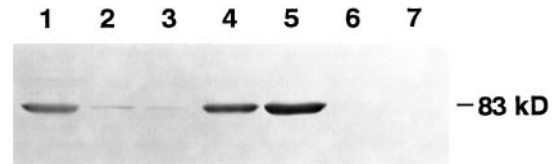
Figure 6. Western-blot analysis of isoamylase in endosperm of various *sugary-1* mutants and their parent cvs at the late-milking stage. The isoamylase preparation was partially purified as in Figure 5A, and 10 μL each of the sample was applied on the SDS-polyacrylamide (10%) gel, followed by immunoblot detection with polyclonal antibodies raised against purified isoamylase from developing rice endosperm (Fujita et al., 1999). Lane 1, cv Kinmaze; lane 2, EM-5; lane 3, EM-41; lane 4, EM-273; lane 5, cv Taichung-65; lane 6, EM-914; lane 7, EM-935.

in the isoamylase gene, as in the maize *sugary-1* mutation (James et al., 1995).

DISCUSSION

The Molecular Mechanism for *sugary-1* Mutations

The molecular mechanism for the *sugary-1* mutation has been most extensively studied in maize endosperm. James et al. (1995) first succeeded in isolating the *Sugary-1* gene from maize by transposon tagging, and recently demonstrated that the gene encodes an isoamylase-type starch debranching enzyme of 79 kD (Rahman et al., 1998). Beatty et al. (1999), on the basis of enzymic characteristics, suggested that the SU1 protein corresponds to the isoamylase II form of maize endosperm previously reported by Doehlert and Knutson (1991). These authors found that isoamylase proteins in maize endosperm are composed of I and II forms distinguishable by anion-exchange chromatography, although form I is greatly labile and remains to be characterized. Rahman et al. (1998) and Beatty et al. (1999), however, both observed only a single isoamylase peak in SDS-PAGE and Q-Sepharose anion-exchange chromatograms, and anti-SU1 failed to detect any protein in maize endosperm other than the SU-1 isoamylase itself. Since no specific assay of isoamylase in the crude enzyme preparation is available, it is most probable that maize isoamylase exists in a single form, although it is possible that the isoamylase I form, which has a structure and properties distinct from those of the type II isoamylase, can partially account for the activity of maize endosperm isoamylase.

The *Sugary-1* gene of rice plants has been mapped on chromosome 8 (Yano et al., 1984). It was previously shown that the genes coding for isoamylase and pullulanase are present as single genes and are localized on chromosomes 8 (Fujita et al., 1999) and 4 (Nakamura et al., 1996a), respectively. However, it has not been proven whether the *Sugary-1* gene is identical to the isoamylase gene or if the two are different genes closely located on chromosome 8. The present study demonstrates that the isoamylase activity is lacking or extremely low in the endosperm of all *sugary-1* mutants tested (Fig. 5). It is noteworthy that in EM-273 and its parent cultivar, there exists protein bands of similar sizes that react with equal intensities to antibodies raised against purified isoamylase from rice endosperm

(Fig. 6). This suggests that in the mutant EM-273, isoamylase has been modified, thus losing its activity, and therefore is defective. Since the isoamylase protein in EM-5 is detectable to some extent (Fig. 6), it is possible that the isoamylase protein is also modified, probably at a site different from that in EM-273. These results indicate that the *Sugary-1* gene is the isoamylase gene in rice. The greatly reduced or nil level of isoamylase in EM-914, EM-935, and EM-41 is in agreement with its extremely low or nil activity (Figs. 5 and 6), suggesting that the isoamylase gene in these lines is mutated at the transcriptional level, e.g. defective at the promoter region of the gene.

Effects of *sugary-1* Mutations in the Activities of Isoamylase and Pullulanase: Involvement of Pullulanase in *sugary-1* Phenotypes

Biochemical analyses of *sugary-1* mutants indicate that both types of starch debranching enzymes, isoamylase and pullulanase, are either missing or greatly reduced in the endosperms of maize (Pan and Nelson, 1984; Rahman et al., 1998) and rice (Nakamura et al., 1992, 1996b, 1997, and the present study). It was postulated that the inhibition of pullulanase activity in maize mutants occurs not at the transcriptional but at the posttranscriptional level, since the pullulanase protein is barely detectable despite the normal level of pullulanase mRNA transcript in maturing maize mutant endosperm (Beatty et al., 1999). Protein-to-protein interaction between isoamylase and pullulanase was further assumed, although it has not been directly detected by the yeast two-hybrid system (A.M. Myers, personal communication).

The *Sugary-1* mutation in rice induces the endosperm to differentiate into two distinct regions: iodine-stained starch region and iodine-nonstained phytoglycogen region. The severity of mutation is reflected by the extent of the phytoglycogen region (Nakamura et al., 1997; Fig. 1). Our previous work indicated that the reduction of pullulanase activity in the endosperm only correlates with the extent of the mutated phenotype (Nakamura et al., 1997). Results of the Red Pullulan film test in the present study further demonstrates that the activity of pullulanase in the phytoglycogen region is significantly lower than that in the starch region of the mutated endosperm (Fig. 4). When localization of pullulanase was done immediately after the phytoglycogen and starch regions were actually separated, the activity of pullulanase on soluble protein basis is significantly lower (about 33%) in the phytoglycogen region than in the starch region of EM-5 endosperm (Table I). These results are consistent with the idea that pullulanase is also involved in *sugary-1* phenotype.

The *sugary-1* mutation not only affects isoamylase levels but also pleiotropically affects the pullulanase level. At present, the mechanism of how the level of pullulanase is reduced in inner endosperm cells compared with the outer cells from some *sugary-1* mutant lines such as EM-5 and EM-935 is unknown. Because rice endosperm cells develop centrifugally, it is possible that the expression of pullulanase ceases earlier in the inner cells than in the peripheral cells, assuming that pullulanase expression is restricted to young endosperm cells. Alternatively, the extent of expression of pullulanase is more severely reduced in inner endosperm cells than in outer cells in an unidentified manner.

The present study reveals that isoamylase activity is dramatically reduced in the endosperms of all rice *sugary-1* mutant lines tested (Fig. 5), a reduction more pronounced than that in the pullulanase activity. The isoamylase activity in EM-41, which possesses the highest activity among the *sugary-1* mutants, is at most one order lower than that in the parent cv Kinmaze (Fig. 5), while the pullulanase activity in EM-914, which has the lowest activity among the mutants, exhibits about 15% of the activity in parent cv Taichung-65 (Nakamura et al., 1997). This strongly suggests that the lesion of isoamylase is essential for *sugary-1* mutations of rice. However, it should be pointed out that the remaining activity of isoamylase is not necessarily related to the severity of the *sugary* phenotype.

First, although isoamylase activity is nil or extremely low in the moderately mutated EM-5, the activity in the severely mutated EM-41 is markedly higher than that in EM-5 (Fig. 5, A and B). Figure 5C further shows that the level of isoamylase activity in the starch region of EM-5 endosperm is markedly lower than that in the phytoglycogen region of EM-41 endosperm. These results indicate that the extent of the *sugary* phenotype is not correlated with the reduced isoamylase activity. Thus, there are other factors affecting phytoglycogen synthesis when isoamylase is absent or present at a low level. The present results cannot be explained by the idea that a low isoamylase activity exclusively results in the accumulation of phytoglycogen instead of amylopectin.

Second, isoamylase activity in EM-5 is practically missing in the starch region of the endosperm (Figs. 1A and 5C). The fact that the pullulanase activity is higher in the starch region of EM-5 endosperm than in the phytoglycogen region (Fig. 4; Table I) suggests that the high pullulanase activity is responsible for the formation of amylopectin-like polyglucan instead of phytoglycogen in the starch region (Fig. 3, A and B). Pullulanase can therefore functionally compensate for the lack of isoamylase activity. We conclude that both types of starch debranching enzymes, isoamylase and pullulanase, are involved in the construction of amylopectin fine structure in rice endosperm.

The Biochemical Mechanism for *sugary-1* Mutations

Although two distinct models have been proposed concerning the mechanism for the role of starch debranching enzyme in amylopectin biosynthesis in plant tissues (Ball et al., 1996; Zeeman et al., 1998), we cannot figure out at present how these enzymes contribute distinctly or coordinately to amylopectin structure. Both enzymes exhibit different substrate specificities and kinetic properties for various polyglucans. Isoamylase can efficiently debranch glycogen and phytoglycogen but cannot attack pullulan, while the reverse is true for pullulanase (Nakamura, 1996; Rahman et al., 1998; Fujita et al., 1999), indicating that the preference for modes of branching of polyglucans and the accessibility to highly branched zones of polyglucans differ between the two enzymes. In bacteria, the minimum num-

ber of branched molecules required for debranching by isoamylase differs from the number required for pullulanase (Lee and Whelan, 1971). This suggests that isoamylase and pullulanase play distinct roles in amylopectin biosynthesis, although it is also possible that the two enzymes can complement the roles of the other to some extent.

It is known that α-1,6 branches with the span of one to two glucans from their neighbors account for approximately 35% of the total branches in the amylopectin molecule (Kainuma and French, 1970). Each cluster has a fixed size of about 9 nm in most plant species (Jenkins et al., 1993). Thus, branches are not evenly distributed along the semicrystal lamellae of the amylopectin molecule, but rather are localized at the neck of the cluster, and bunches of branches within a cluster are regularly repeated along the whole amylopectin molecule. This highly organized structure of amylopectin needs to be synthesized by controlled and coordinated actions of branching enzymes, debranching enzymes, and starch synthases.

An apparent discrepancy in the modes of the *sugary* mutation among plant species remains to be elucidated. Both isoamylase and pullulanase appear to be involved in the *sugary* phenotype in endosperms of maize (Rahman et al., 1998; Beatty et al., 1999) and rice (Nakamura et al., 1997; present study), whereas deficiency in isoamylase alone is responsible for phytoglycogen production in Arabidopsis leaves (Zeeman et al., 1998) and *C. reinhardtii* cells (Mouille et al., 1996). One of the simplest explanations for this discrepancy is that there may exist some differences in the mechanism for amylopectin biosynthesis between storage tissues and photosynthetic tissues, a possibility that could be related to the well-known difference between storage starch and assimilatory starch in terms of amylopectin fine structure and starch granule structure (Matheson, 1996; Tomlinson et al., 1997).

The existence of a number of rice *sugary-1* mutants with varying severity in phenotype raises the following questions. First, is the amylopectin fine structure altered continuously or step-wise in accordance with the changes in the activities of debranching and other enzymes? Figure 3 clearly shows that phytoglycogen exhibits a similar pattern in chain length distribution irrespective of the mutant lines tested, and the polyglucans in the starch regions have a common distinct structure with a chain length distribution significantly different from the amylopectin in wild-type endosperm (cv Kinmaze). These results indicate that when isoamylase and pullulanase activities are low or nonexistent, polyglucans in rice endosperm tend to be either of the two distinct structures, namely phytoglycogen or the so-called *sugary*-amylopectin. Second, is there any difference in the fine structure of phytoglycogen and glycogen on the basis of chain length distribution? The small shoulder at DP values around 17 to 19 found in the phytoglycogen curve for the chain length distribution (Fig. 3B) is absent in glycogen (data not shown). This suggests that there is still a trace of a cluster structure in the phytoglycogen molecule. More precise analysis will be needed to clarify this possibility.

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