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Effect of high temperature on fine structure of amylopectin in rice endosperm by reducing the activity of the starch branching enzyme

Huawu Jiang, Weimin Dian, Ping Wu*

State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou, 310029, PR China

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Abstract

Rice (*Oryza sativa* L.) grain quality is affected by the environmental temperature it experiences. To investigate the physiological molecular mechanisms of the effect of high temperatures on rice grain, a non-waxy indica rice was grown under two temperature conditions, (29/35 °C) and (22/28 °C), during the ripening stage in two phytotrons. The activities and gene expression of key enzymes for the biosynthesis of amylose and amylopectin were examined. The activity and expression levels of soluble endosperm starch synthase I were higher at 29/35 °C than that at 22/28 °C. In contrast, the activities and expression levels of the rice branching enzyme1, the branching enzyme3 and the granule bound starch synthase of the endosperm were lower at 29/35 °C than those at 22/28 °C. These results suggest that the decreased activity of starch branching enzyme reduces the branching frequency of the branches of amylopectin, which results in the increased amount of long chains of amylopectin of endosperm in rice grain at high temperature.

Keywords: Rice (*Oryza sativa* L.); Gramineae; Environmental temperature; Starch synthase; Starch branching enzyme; Starch biosynthesis; Amylose; Amylopectin

1. Introduction

The starch of non-waxy rice (*Oryza sativa* L.) endosperm tissue contains two types of polysaccharides, amylose and amylopectin. Both the ratio of amylose to amylopectin, and the fine structure of amylopectin, are important factors in determining the nutritional quality of rice (Asaoka et al., 1985; Reddy et al., 1993). Amylose is a linear molecule containing α (1→4)-linked glucose units with a small number of branches. As in other cereals, the amylose in endosperm of rice grain is synthesized by a granule-bound starch synthase (GBSS), a product of the *waxy* gene (Sano, 1984). In contrast, amylopectin is a highly branched biopolymer consisting of linear chains of α (1,4)-linked glucose residues joined together by α (1,6)-linkages. Within the granule, the chains are thought to be arranged in clusters at intervals of 9 nm within which chains are associated to form double helices. These helices are packed in ordered arrays to give concentric crystalline lamellae, and the distribution of branch lengths in amylopectin is important in determining the crystalline nature of the starch granule and the physical properties of the starch. The specific pattern of chain-length profiles in amylopectin results from the relative activites of distinct isoforms of soluble starch synthase (SSS) and starch branching enzyme (SBE) in starch biosynthetic organs (Smith et al., 1997; Ball et al., 1998; Smith, 1999; Myers et al., 2000).

At least four distinct starch synthase (SS) isoforms can be defined based on their amino acid sequences, namely the granule-bound starch synthase (GBSS) and three isoforms of soluble starch synthase SSSI, SSSII, and SSSIII (Cao et al., 1999; Smith, 1999; Li et al., 2000; Myers et al., 2000). Mutations in the maize gene *du1* (SSSIII) and the *Chlamydomonas reinhardtii* gene *sta-3* (SSSII) could cause significant changes in chain length distribution of amylopectin (Fontaine et al., 1993; Gao et al., 1998). Pea *rug5* mutations affecting SSSII significantly alter starch granule structure and chain length distribution (Craig et al., 1998). Simultaneous depletion of SSSII and SSSIII isoforms in potato leads to

* Corresponding author. Tel.: +86-571-8697-1130; fax: +86-571-8697-1323.
E-mail address: docpwu@cls.zju.edu.cn (P. Wu).

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accumulation of grossly modified amylopectin with an altered branching pattern (Edwards et al., 1999; Lloyd et al., 1999). Individual SSS, therefore, can have specific effects on the fine structure of amylopectin. On the other hand, the individual SSS exhibits different thermostability behaviour. A decreased activity of SSS in wheat was observed at high temperature (Keeling et al., 1993), whereas the SSS activity in maize and rice remained high at high temperatures up to 37 °C (Cao et al., 2000; Cheng et al., 2001). In maize, SSSI exhibits a higher optimum temperature than SSSIII, and it is heat stable at 42 °C unlike the SSSIII/DU1, which is labile at this temperature (Cao et al., 2000).

SBEs catalyze the cleavage of α (1,4) linkages and release the reducing end to α-C6 hydroxyl, producing a new α (1,6) linkage. The SBEs from various higher plants can be divided into two types, namely BEI and BEII from cereals, or the A-type and B-type from pea (Smith et al., 1997). Three isoforms of rice branching enzyme (RBE), RBE1, RBE3 and RBE4, are present in rice endosperms. RBE3 and RBE4 fall into class BEII, whereas RBE1 falls into class BEI, which plays a central role in transferring longer chains (Yamanouchi and Nakamura, 1992; Mizuno et al., 1992, 1993, 2001). SBE-deficient mutants have been isolated from maize (Baba and Arai, 1984; Stinard et al., 1993), rice (Mizuno et al., 1993; Nishi et al., 2001) and pea (Bhattacharyya et al., 1990). In maize and rice, they have been designated as *amylose-extender* (*ae*) mutants and the average chain length of amylopectin in *ae* endosperm is significantly longer than that of normal amylopectin (Baba and Arai, 1984; Mizuno et al., 1993; Nishi et al., 2001). In pea embryos, the branched starch biopolymer is greatly reduced in the wrinkled (*rr*) line as compared to that in wild-type (Bhattacharyya et al., 1990, 1993). These observations suggest that mutation of genes for SBE leads to alterations in the fine structure of amylopectin and the function of one isoform of SBE cannot be complemented by the other isoforms.

It has been suggested that higher environmental temperature decreases amylose content in endosperm starches of non-waxy rice plants, as a result of decreased activity of GBSS, where gene expression in rice grains is regulated by temperature at the transcriptional and post-transcriptional level (Asaoka et al., 1985; Hirano and Sano, 1998; Larkin and Park, 1999). Higher environmental temperature could also increase the amount of long B chains of amylopectin and decrease short B chains (Asaoka et al., 1985). However, the mechanisms by which temperature affects the fine structure of amylopectin of rice grain are unclear so far. In this study, the major isoforms of amylopectin-synthesizing enzymes, SSS and SBE were investigated in order to understand the biochemical basis of the effects of high temperature on the fine structure of amylopectin.

2. Results and discussion

2.1. Effect of high temperature on rice grain-filling duration

As shown in Fig. 1, the dry weight of hulled rice (Zhe733, indica) grain developed at 29/35 °C increased rapidly within 10 days after flowering (DAF), whereas at the lower temperature (22/28 °C), grain-filling duration extends to 20 days. Because of the difference in grain-filling rate at different temperatures, the early milky stage, the mid-milky stage and the dough stage were observed at 4, 7 and 10 DAF at 29/35 °C, in contrast to 7, 11 and 15 DAF, at 22/28 °C. The periods of extensive grain filling were within 5–9 DAF at 29/35 °C and 7–15 DAF at 22/28 °C, respectively.

2.2. Effect of high temperature on amylose content and the fine structure of amylopectin in rice endosperms

Some characteristics of isoamylase-debranched materials of endosperm starches of the non-waxy indica rice, Zhe733, grown under different temperatures after flower-

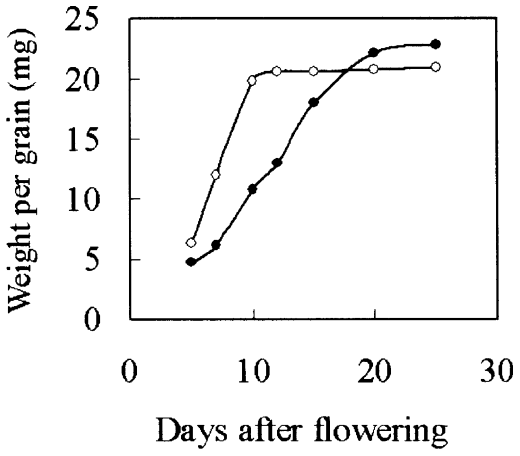

Fig. 1. Time courses of dry weight of hulled rice grain developed under different ambient temperatures after flowering. Values are mean of at least 30 grains. (○) 29/35 °C, (●) 22/28 °C.

Table 1
Properties of isoamylase debranched materials of endosperm starches from rice plants grown at high temperature (29/35 °C) and low temperature (22/28 °C) after flowering

Temperature	Distribution of starch components (%)				Fr. III/ Fr. II
	Fr. I $\lambda_{max} \geq$ 620 nm $\overline{CL} \geq 64$	Inf 620 nm $> \lambda_{max} >$ 600 nm $64 > \overline{CL} > 58$	Fr. II 600 nm $\geq \lambda_{max} \geq$ 525 nm $58 \geq \overline{CL} \geq 22$	Fr. III $\lambda_{max} <$ 525 nm $\overline{CL} < 22$	
22/28 °C	24.2	2.4	22.4	51.0	2.28
29/35 °C	18.2	2.3	27.7	51.8	1.80

$\overline{CL}$-average chains lengths.

ing are summarized in Table 1. Fraction I, II and III, were defined according to the wavelengths of absorption maxima (λ_{max}) of their respective iodine–carbohydrate complexes. Fr I, whose iodine–carbohydrate complexes have a λ_{max} longer than 620 nm, was regarded as amylose, whereas Fr. II (600 nm $\geqslant \lambda_{max} \geqslant$ 525 nm) and Fr. III ($\lambda_{max} <$ 525 nm) correspond to the linear unit-chains of amylopectin. The amylose content (Fr. I) in rice endosperms was reduced by 25% at high temperature, while Fr. II and Fr. III showed peaks with average chain lengths of ~38 and ~16, respectively. The ratio of Fr. III to Fr. II was higher at lower temperature than at higher temperature, which suggested that the amylopectin of the endosperm grown at 29/35 °C contained more long chains than that grown at 22/28 °C. This result is consistent with a previous report (Asaoka et al., 1985).

2.3. Effect of high temperature on the activity and the gene expression of SSSI in developing rice endosperms

As shown in Fig. 2A, there was no overt difference in the total soluble starch synthase activity in the endosperm across the main period of grain filling in the two cultivars. In rice seeds (10–15 DAF), the proteins corresponding to soluble starch synthase are products of *SSSI* gene (Baba et al., 1993). The present results from native polyacrylamide gels electrophoresis (PAGE)/ activity staining indicated that the crude extract of endosperm yielded three bands of SSS (Fig. 3A). Two weak bands should be a class II SSS, since they were expressed earlier during the development of rice endosperm, and the major band should be SSSI, since it was expressed subsequently. The amount of SSSI was very low at the early-milky stage at 22/28 °C (lane 6 in Fig. 3A). Quantitative analysis of the bands using the program ImageQuant (Molecular Dynamics) indicated that the activity of SSSI in the endosperm of rice plants grown at 29/35 °C was about 10–22% higher than that at 22/28 °C across the main grain filling stage. Northern blot analysis of *SSSI* is consistent with this result (Fig. 4). Our recent research indicated that SSSI in rice endosperms is also granule-bound, and the protein amount of the granule-bound SSSI based on the dry weight of starches is also higher at 29/35 °C than that at 22/28 °C (unpublished data). These results suggest that the higher activity of SSSI complements the decreased activities of SSSII (unpublished data) and GBSS (Asaoka et al., 1985), and maintains the elongation of the chains of amylopectin in rice endosperms at high temperature.

2.4. Effect of high temperature on the activity and gene expression of GBSS in developing rice endosperms

The activity of GBSS is necessary to generate amylose (Smith et al., 1997; Ball et al., 1998). As shown in Figs. 2C and 4, the activities and the gene expression of

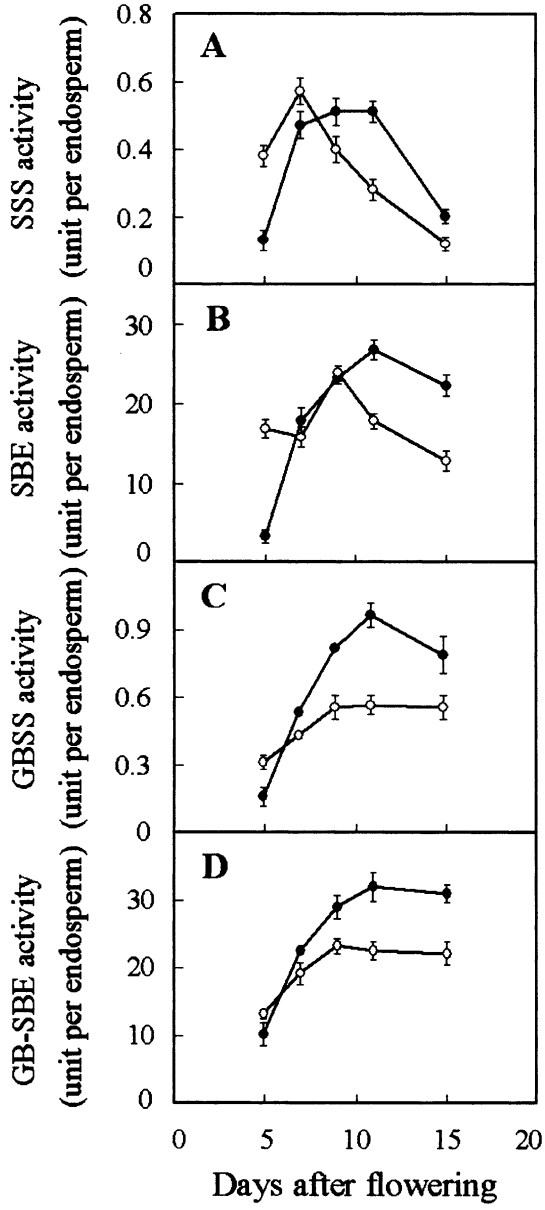

Fig. 2. Comparison of activities of soluble starch synthase (SSS) (A), soluble starch branching enzyme (SBE) (B), granule-bound starch synthase (GBSS) (C), and granule-bound starch branching enzyme (GB-SBE) (D) in rice endosperm developed at 22/28 °C and 29/35 °C after flowering. (○) 29/35 °C, (●) 22/28 °C. The data represent averages of three repeats of the analysis with the mean standard deviation.

GBSS at 29/35 °C were decreased in endosperms compared with those at 22/28 °C. The present results support the previous reports, that the amylose content is mainly affected by the activity of GBSS in rice endosperms at high temperature (Sano et al., 1985; Umemoto et al., 1995; Hirano and Sano, 1998).

2.5. Effect of high temperature on the activity and expression level of SBE in developing rice endosperms

As shown in Fig. 2B, the total activity of soluble SBE across the main period of grain filling at 29/35 °C was

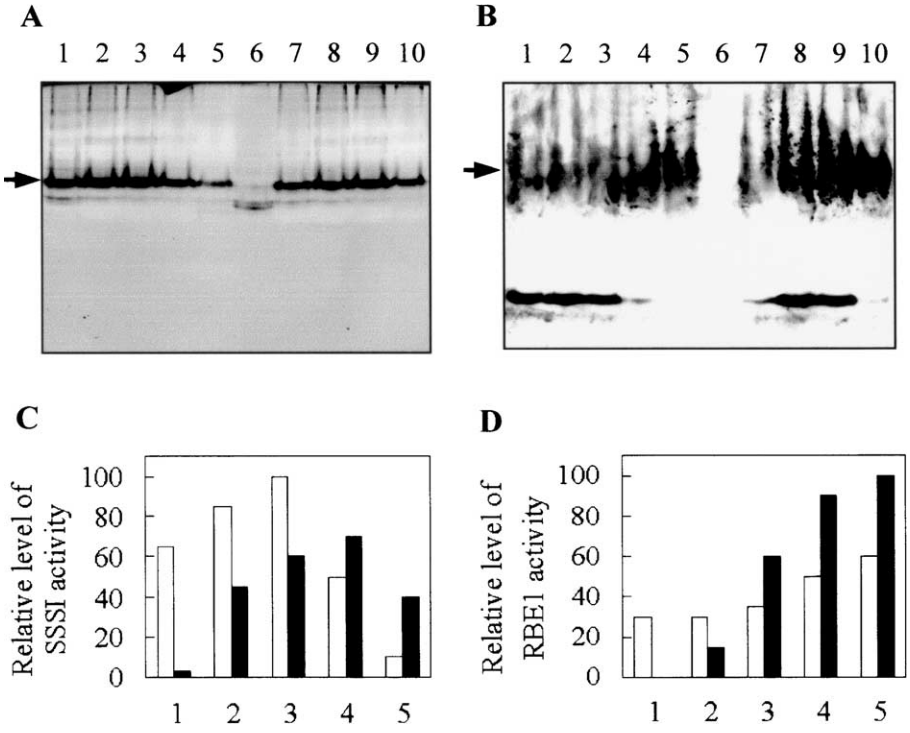

Fig. 3. Zymogram analysis of SSS and SBE of rice endosperm developed at 22/28 °C and 29/35 °C after flowering. (A) After separation of SSS isoforms by native-polyacrylamide gel electrophoresis (PAGE), enzyme reactions were performed with the addition of 0.1% (w/v) potato amylopectin and 0.5 M citrate. One major band of SSSI (the arrow indicated) and two isoforms of Class II were observed. (B) After separation of SBE isoforms by native-PAGE, enzyme reactions were performed either with the addition, or in the absence (data not shown) of, exogenous rabbit muscle phosphorylase a. One major band of RBE1 and two isoforms of RBE3 and RBE4 were observed. The band of RBE1 tended to be indistinct and broad (the arrow indicated). Lanes 1–5 were loaded with 20 μl of the crude extract of endosperm at 5, 7, 9, 11, and 15 DAF, respectively, grown at 29/35 °C; lanes 6–10 were loaded with 20 μl of the crude extract of endosperms at 5, 7, 9, 11, and 15 DAF, respectively, grown at 22/28 °C. (C) and (D) Correlation of SSSI activity and RBE1 activity. Relative band areas were determined by using ImageQuant program. The maximum activity defined as 100%. Lanes 1–5: 5, 7, 9, 11, and 15 DAF. (□) 29/35 °C, (■) 22/28 °C.

lower than that at 22/28 °C. The native PAGE/activity staining analysis indicated that the crude extract of endosperm yielded one major band of RBE1 with lower electrophoretic mobility and the other two isoforms, RBE3 and RBE4, with higher mobility. The band of RBE1 tended to be indistinct and broad, and showed very low activity at the early-milky stage at 22/28 °C (lane 6 in Fig. 3B), and an endogenous phosphorylase apparently overlapped with a higher mobility band of RBE. Quantified using the program ImageQuant, the activity of RBE1 in rice endosperms grown at 29/35 °C was about 60–70% of that at 22/28 °C across the main period of grain filling (Fig. 3B and D). Northern blot analysis using total RNAs from developing seeds revealed that the level of *RBE1* mRNA quantified using the program ImageQuant was reduced about 30% in the endosperm at 29/35 °C relative to that at 22/28 °C across the main period of grain filling (Fig. 4). It has been revealed recently that RBE3 is also starch granule-bound in rice grains, and the protein amount of the bound RBE3 based on the dry weight of starches is lower at 29/35 °C than those at 22/28 °C (unpublished data). The activity of granule-bound SBE (RBE3) (Fig. 2D) and the content of its transcript (Fig. 4) were also reduced in the endosperm of rice grown at 29/35 °C compare to that at 22/28 °C. The amount of transcript of RBE4, which expressed earlier during the developing of rice endosperm, was slightly influenced by environmental temperature (Fig. 4). In conclusion, SBE activity in developing rice endosperm was decreased by the reduced expression of RBE1 and RBE3 at high temperature.

In *ae* maize and rice endosperms, and *rr* pea embryos, the average chain length of amylopectin is significantly longer than that of normal amylopectin (Baba and Arai, 1984; Bhattacharyya et al., 1990, 1993; Mizuno et al., 1993; Nishi et al., 2001). The temperature sensitivity response in vitro was not indicative of an overt change in rice SBE activity at high temperature up to 37 °C (Cheng et al., 2001). These results suggest that the decreased activity of SBE in rice endosperms may decrease the branching frequency of amylopectin, thus increasing the proportion of long chains (decreased the ratio of Fr. III to Fr. II) of amylopectin in rice grains grown at higher temperature.

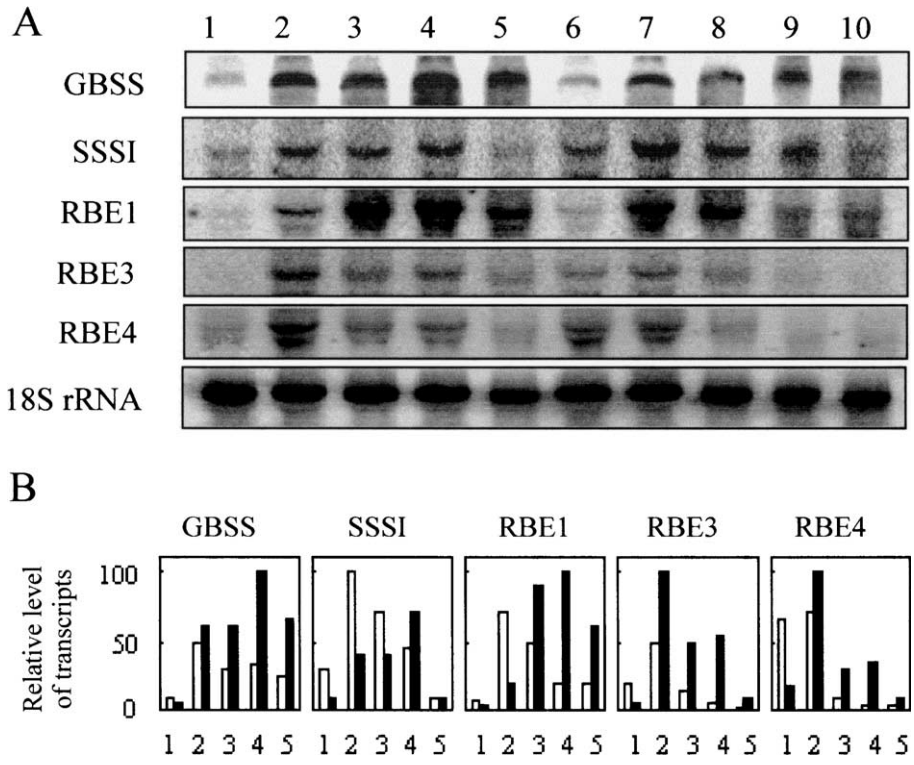

Fig. 4. (A) Gel blot analysis of total RNA from developing endosperms. Lanes 1–5 were loaded with 20 μg of the total RNA of grains at 3, 5, 8, 11, and 14 DAF, respectively, grown at 22/28 °C; lanes 6–10 were loaded with 20 μg of the total RNA of grains at 3, 5, 8, 11, and 14 DAF, respectively, grown at 29/35 °C. The RNAs were fractionated on a formaldehyde-agarose gel, blotted, and probed with the cDNA clones from MAFF DNA Bank (Names of the EST clones were: GBSS, E10479; SSSI, E1430; RBE1, E10727; RBE3, E11576; and RBE4, S14669, respectively. These clones were confirmed by sequencing). Minor loading differences were calibrated by hybridization of the 18S rRNA with a tomato 18S rDNA probe on the same blot. (B) Quantification of the RNA expression data shown in (A). Relative band areas were determined by using ImageQuant program. Each of the five gene expression levels at each stage was normalized against the 18S data, and the maximum expression level was defined as 100%. Lanes 1–5: 3, 5, 8, 11, and 14 DAF. (□) 29/35 °C, (■) 22/28 °C.

3. Concluding remarks

Results from enzyme assays, native PAGE/activity staining, and Northern blot analyses showed that temperature modulated the gene expression levels and activities of SSS and SBE in rice endosperms. The lower amylose content in rice endosperms at high temperature may be mainly attributed to the reduced activity of GBSS. The increased activity of SSSI, which is the predominant SSS in rice grains, maintained the elongation of the A and B chains of amylopectin; while the decreased activity of RBE1 and RBE3, the predominant of soluble SBE and granule-bound SBE in rice grain, lowered the branching frequency of amylopectin. Therefore, the diverse changes in levels of SSS and SBE may result in the increased amount of long chains of amylopectin in rice grain at high temperature.

4. Experimental

4.1. Plant material

Rice (*O. sativa* L. Indica. Zhe733) was grown in the greenhouses at Zhejiang University (Hangzhou, PR China) until anthesis. One day after flowering, plants were moved into growth chambers maintained at 22/28 °C (night/day) or 29/35 °C under natural daylight. The grains were marked on the hull at the day of flowering. The grains located directly on the second to fourth upper-primer primary branches (except the two grains from apex) were used in this study. Rice grains from ten individual panicles were harvested and stored at −75 °C until used. The grains were harvested several times during the maturation process. The remaining panicles were allowed to ripen and harvested at maturity for amylose content and amylopectin fine structure determination (Umemoto et al., 1995).

4.2. Analysis of α-polyglucan structure

The embryo and pericarp were removed from mature grains (30 grains), and the endosperms were treated and debranched with isoamylase according to the methods described by Asaoka et al. (1985). The debranched sample was dissolved in 1 M NaOH (1 ml) at 30 °C for 2 h, then 1 ml of distilled water was added. The preparation was applied onto a Sephadex G-75 column which had been equilibrated with 0.02 M NaOH containing

0.2% (w/v) NaCl. The debranched α-1, 4-glucans were eluted with the same solution at a flow rate of 0.2 ml min^{-1} (ÄKTA, P-920, Amersham Pharmacia Biotech). Fractions were collected at 2-ml intervals and each fraction was neutralized with 1 M HCl. The range of fractions I, II and III, was divided according to the wavelengths of absorption maxima (λ_{max}) of absorption spectra of iodine–carbohydrate complexes in each tube (Beckman-DU640): Fr. I, $\lambda_{max} \geqslant 620$ nm; Inf, 620 nm $> \lambda_{max} >$ 600 nm; Fr. II, 600 nm $\geqslant \lambda_{max} \geqslant 525$ nm; and Fr. III, $\lambda_{max} < 525$ nm. The carbohydrate content in each fraction was measured by the phenol-sulfuric acid method (Rao and Pattabiraman, 1989). Reducing end groups were determined by the potassium ferricyanide method (Porro et al., 1981). The average chain lengths ($\overline{CL}$) were calculated from the amounts of carbohydrate and the number of reducing ends (Asaoka et al., 1985).

4.3. Preparation of enzymes

All procedures were performed at 0–4 °C. Ten hulled grains were removed from embryo and pericarp, and hand-homogenized with a glass homogenizer in 2 ml of solution containing 50 mM HEPES–NaOH (pH 7.4), 4 mM $MgCl_2$, 2 mM EDTA, 50 mM 2-mercaptoethanol and 12.5% (v/v) glycerol. The homogenate was centrifuged at 10,000 *g* for 10 min, and the pellet was washed (2 ml×2) with the same buffer. The resulting supernatants were used for the preparation of enzymes, and the pellets were resuspended in the same buffer (2 ml) and used for the assay of GBSS and granule-bound starch branching enzyme (GB-SBE) (Nakamura et al., 1989; Yamanouchi and Nakamura, 1992; Umemoto et al., 1995).

4.4. Enzyme assays

4.4.1. Starch synthase (EC 2.4.1.21)

The assay was conducted in a reaction mixture (280 μl) containing 50 mM HEPES–NaOH (pH 7.4), 1.6 mM ADP-glucose, 0.7 mg amylopectin, 15 mM DTT, and enzyme preparation. Twenty minutes after the start of the reaction at 30 °C, the enzyme was inactivated by placing the mixture in a heating-block at 98 °C for 1 min. A solution (100 μl) of 50 mM HEPES–NaOH (pH 7.4), 4 mM PEP, 200 mM KCl, 10 mM $MgCl_2$, and pyruvate kinase (1.2 units) was added to the mixture, and incubated for 30 min at 30 °C. The resulting solution was heated in a 98 °C heating-block for 1 min, and then subjected to centrifugation at 10,000 *g* for 5 min. The supernatant (300 μl) was mixed with a solution (200 μl) of 50 mM HEPES–NaOH (pH 7.4), 10 mM glucose, 20 mM $MgCl_2$, and 2 mM NADP. The enzymatic activity was measured as the increase in absorbance of 340 nm after the addition of hexokinase (1 μl, 1.4 units) and glucose 6-phosphate dehydrogenase (1 μl, 0.35 unit) (Nakamura et al., 1989; Nishi et al., 2001). One unit of enzymatic activity was defined as the formation of 1 nmol ADP in 1 min.

4.4.2. Branching enzyme (EC 2.4.1.18)

Assays were conducted at 30 °C in a reaction mixture (200 μl) containg 50 mM HEPES–NaOH (pH 7.4), 5 mM glucose-1-P, 1.25 mM AMP, phosphorylase a (54 units), and enzyme preparation. The reaction was terminated by addition of 1 M HCl (50 ml). The solution was mixed with dimethylsulfoxide (500 μl) and iodine solution (700 μl, 0.1% I_2 and 1% KI). The enzymatic activity was assayed spectrophotometrically at 540 nm. One unit of enzymatic activity was defined as the amount causing an increase in absorbance of one unit at 540 nm in 1 min (Nakamura et al., 1989; Yamanouchi and Nakamura, 1992).

4.5. Native gel electrophoresis and activity staining

Enzyme extracts (20 μl in each lane) were separated on gels prepared with 7.5% (resolving gel) and 2.5% (stacking gel) polyacrylamide with electrophoresis was carried out at 100 V and at 4 °C (Hoefer miniVE electrophoresis systems, Amersham Pharmacia Biotech). Starch synthase activity was detected after incubation of the gel in 50 mM Tricine–NaOH (pH 8.5), 0.5 M sodium citrate, 25 mM potassium acetate, 2 mM EDTA, 2 mM DTT, 0.1% (w/v) potato amylopectin and 1 mM ADP-glucose at 30 °C for 4 h. Following incubation the gels were stained with iodine solution (Buléon et al., 1997; Cao et al., 1999). For assays of branching enzyme, after electrophoresis, the gel was rinsed with 20 ml of 50 mM HEPES–NaOH buffer (pH 7.0) and 10% glycerol for 30 min at 4 °C, and then incubated for 2 h at 30 °C in 20 ml of SBE reaction mixture, which consisted of 50 mM HEPES–NaOH buffer (pH 7.0), 50 mM glucose-1-P, 2.5 mM AMP, 10% Glycerol, and rabbit muscle phosphorylase a (about 60 units). For assays of endogenous phosphorylase activity in sample, rabbit muscle phosphorylase a was omitted from the reaction mixture. After incubation, the gel was placed in the iodine solution (Yamanouchi and Nakamura, 1992).

4.6. Northern blot analysis

Total cellular RNAs (20 μg) prepared from endosperms were separated on 1.2% formaldehyde-agarose gels and transferred onto Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The blots were probed by DNA fragments that had been labeled with [α-^{32}P]dCTP, using the random-priming DNA labeling method. After washing, the blots were dried and analyzed using Typhoon-8600 (Amersham Pharmacia Biotech).

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