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Thermal and Pasting Properties, Morphology of Starch Granules, and Crystallinity of Endosperm Starch in the Rice SSI and SSIIa Double-Mutant

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Abstract: Starch is glucose polymer linked by α -1,4 and α -1,6 glucosidic bonds. The balance of activity between starch synthases (SSs) and branching enzymes (BEs) is important for formation of amylopectin structure. SSI and SSIIa isozymes account for 60 and 30%, respectively, of soluble SS activity in developing rice endosperm. Complete loss of both SSI and SSIIa activities induces sterility. By contrast, a double mutant (*ssI^L/ss3a*) generated by crossing the leaky *ssI* mutant, whose SS activity is approximately ca. 16% of the wild type, and the *ss3a* null mutant is fertile. Although there is only a significant residual SS activity in the developing endosperm of *ssI^L/ss3a*, the yield and growth of the double-mutant line is sufficient. We analyzed the amylopectin chain-length distribution, thermal and pasting properties, morphologies of starch granules and amyloplasts, and crystallinity of endosperm starch in *ssI^L/ss3a* and the parental mutant lines. The chain-length distribution pattern of *ssI^L/ss3a* was similar to that of *ss3a* and there was an additive effect of the reduction of SSI activity on the chain-length of amylopectin in the *ss3a* background. These changes in the amylopectin chain-length distribution were considered to increase the gelatinization temperature of the starch. The gelatinized starch viscosity of the double-mutant and *ss3a* lines was very low. Most *ssI^L/ss3a* starch granules were round-shaped, and the starch granular size was smaller than those of the parental mutant lines and the wild type. The starch crystallinity of *ssI^L/ss3a* was slightly reduced compared with that of the wild type.

Key words: differential screening calorimetry (DSC), scanning electron microscopy (SEM), starch synthase, rapid visco analyzer (RVA), X-ray diffraction

INTRODUCTION

Plants store starch in the forms of amylose and amylopectin. Amylose is a linear carbohydrate joined by an α -1,4 glucosidic linkage, whereas amylopectin has a tandem cluster structure with branched chain residues joined by α -1,6 glucosidic bonds. At least four enzymes are involved in starch biosynthesis, namely ADP-glucose pyrophosphorylase, starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme (DBE).^{1–4)}

SS elongates α -glucans by adding glucose residues from ADP-glucose to the glucan at the non-reducing ends. There are 11 SS isozyme genes in the rice genome, which are classified into the following six groups: SSI, SSII, SSIII, SSIV, SSV and granule-bound starch synthase (GBSS).⁵⁾ SSI and SSIIa account for 60 and 30% of SS activity, respectively, in the soluble fraction of developing rice endosperm.⁶⁾

The rice *ssI* null mutant lacks all SSI activity, but the mutant seed phenotype (seed morphology and seed weight)

is not significantly different from that of the wild type. However, the amylopectin chain-length distribution in *ssI* specifically differed from that of the wild type.⁶⁾ An analysis of the mutant chain-length distribution suggested that SSI generates the DP 8–12 chains from the DP 6–7 chains that emerge from the branch point in the A and B₁ amylopectin chains in the endosperm.⁶⁾

The rice seed phenotype of the *ss3a* null mutant is characterized by a white core, although the seed weight is comparable to that of the wild type.⁷⁾ SSIIa elongates long B_{2–3} amylopectin chains with DP $\geq$ 30. A deficiency of SSIIa causes pleiotropic effects on SSI and GBSSI; enhanced expression of SSI and GBSSI leads to changes in the distribution of chains with DP $\leq$ 20 and a 1.5-fold increase of amylose content, respectively.⁷⁾

To generate a double mutant defective for SSI and SSIIa, we crossed the *ssI* null mutant with the *ss3a* null mutant. However, fertile double-recessive seeds lacking SSI and SSIIa activities were not obtained.⁸⁾ Further studies determined that if one of the genes was heterozygous (*ssIssI/SS3ass3a* or *SSIssI/ss3ass3a*), opaque seeds were obtained. These results suggested that either SSI or SSIIa was essential for starch biosynthesis in rice endosperm, and no other SS isozyme provided complementation.⁸⁾ A fertile double-recessive homozygous mutant line (*ssI^L/ss3a*) was

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Abbreviations: BE, starch branching enzyme; DSC, differential scanning calorimetry; DP, degree of polymerization; GBSSI, granule-bound starch synthase I; RVA, rapid visco analyzer; SEM, scanning electron microscopy; SS, starch synthase.

obtained by crossing the *ss1-leaky* mutant (*ss1^L*), which expresses 16% of the wild-type SSI activity, with the *ss3a* mutant.⁸⁾ The *ss1^L/ss3a* double-mutant line was created in the japonica cultivar Nipponbare background to ensure that SSIIa was inactive and the GBSSI activity was much lower than that in *Indica* cultivars. The *ss1^L/ss3a* seed weight was 86% of that of the wild type and the growth was sufficient although the total remaining soluble SS activity in the *ss1^L/ss3a* double mutant must be < 10% of that of the wild type (Nipponbare). These results suggest that severely limited SS activity (either SSI or SSIIa) is sufficient to produce starch in the rice endosperm. However, the starch traits including physicochemical properties and starch morphologies have not been investigated. Therefore, this study characterized the amylopectin chain-length distribution, thermal and pasting properties, starch granule morphology, and crystallinity of endosperm starch in the *ss1^L/ss3a* double-mutant line.

MATERIALS AND METHODS

Plant materials. The *ss1^L/ss3a*⁸⁾ double-mutant line (#6002) was generated by crossing a leaky mutant for SSI activity (*ss1^L, i2-1*)⁶⁾ with a defective mutant for SSIIa activity (*ss3a, e1*)⁷⁾. The parental cultivar Nipponbare and parental mutant lines (*ss1^L* and *ss3a*) also were used in the experiments. Rice plants were grown during the summer in 2010 (for RVA) and 2013 (for the other analyses) in an experimental paddy field at Akita Prefectural University under natural environmental conditions.

Starch granule purification. Starch granules were prepared from polished rice grains free from the embryos using the cold-alkali method.^{9,10)}

Analytical methods. The chain-length distribution of endosperm α -glucan was analyzed by the capillary electrophoresis methods^{11,12)} with a P/ACE MDQ Carbohydrate System (Beckman Coulters Inc., Brea, USA). The thermal

properties of endosperm starch were determined by differential scanning calorimetry (DSC) (DSC6100, Seiko Instruments Inc., Japan) according to a previously published protocol.⁶⁾ The pasting properties of endosperm starch were determined by a rapid visco analyzer (RVA) (RVA-4, Netport Science PTY Ltd., Warriewood, Australia) using the previously published methods.¹³⁾ Purified starch granules from mature seeds were observed by scanning electron microscopy (SEM) using the previously published methods.^{7,14)} X-ray diffraction was performed as described previously.¹⁵⁾ Endosperm starch crystallinity was determined with respect to an internal standard using the previously published methods¹⁶⁾. Data smoothing and background elimination were calculated using the method¹⁶⁾. Relative starch crystallinity (RSC) was calculated by Eq. (1).

$$\text{RSC} = \text{SD/IS} \quad (1)$$

Where SD and IS are the area of specific diffractograms [the peaks of A-type starch are 15°, 17°, 18°, 20°, and 23° (2 θ)] and area of internal standard, respectively. Relative amylopectin crystallinity (RAC) was calculated by Eq. (2).

$$\text{RAC} = \text{RSC}/(1 - \text{Apparent amylose content}/100) \quad (2)$$

The significant differences between lines were statistically evaluated by *t*-test at *P* < 0.05.

RESULTS AND DISCUSSION

Analysis of amylopectin structure.

Differences in the chain-length distribution patterns among the mutant lines and the wild type (Nipponbare) were analyzed by capillary electrophoresis (Fig. 1). The results obtained in this study were consistent with the previous reports.⁶⁻⁸⁾ For *ss1^L*, the levels of DP 7–12 short chains were

Fig. 1. Differential chain-length distribution plots of double-mutant and parental mutant lines and the wild type (Nipponbare).

Each chart shows one representative data set (one of at least three independent replications using different preparations from different rice seeds of homogeneous plants). The region that contributes to reducing and increasing the gelatinization temperature is shown in blue and red, respectively. (A) *ss1^L*-Nipponbare, (B) *ss3a*-Nipponbare, (C) *ss1^L/ss3a*-Nipponbare, (D) Overlay of three mutant lines.

reduced, whereas the levels of DP 6 and DP 16–21 chains increased compared with those of the wild type (Fig. 1 (A)). For *ss3a*, the levels of amylopectin chains with DP 6–9 and DP 16–19 were reduced, whereas the levels of DP 10–15 increased compared with those of the wild type. A significant reduction of B₂₋₃ amylopectin chains with DP 31–50 was observed for *ss3a* (Fig. 1 (B)). The chain-length distribution pattern of *ss1^L/ss3a* was essentially equivalent to that of *ss3a* (Figs. 1 (C) and 1 (D)), except for the more pronounced reduction of chains with DP 7–9 and increase of chains with DP10–20 than observed for the wild type (Fig. 1 (D)). This indicates that there was an additive effect of the reduction of SSI activity on the chain-length of amylopectin in the *ss3a* background.

Thermal properties of endosperm starch.

Thermal properties (gelatinization temperature and enthalpy) of endosperm starch were measured by DSC (Fig. 2 and Table 1). The DSC curve for Nipponbare, *ss1^L*, and *ss1^L/ss3a* had sharp endothermic peak, whereas that of *ss3a* had a broad peak (Fig. 2). The broad peaks detected at 90–100°C in *ss3a* and *ss1^L/ss3a* starch having high amylose content must be derived from amylose-lipid complex. The peak temperatures of *ss1^L* and *ss3a* endosperm starches were 1.2°C higher and 1.8°C lower than that of the wild type, respectively (Table 1). The peak temperature of *ss1^L/ss3a* endosperm starch was 3.2°C higher than that of the wild type. The gelatinization enthalpy of *ss1^L* and *ss3a* was 1.0 mJ/mg higher and 1.8 mJ/mg lower, respectively, than that of the wild type. The gelatinization enthalpy of *ss1^L/ss3a* endosperm starch was comparable to that of the wild type (Table 1).

Hizukuri¹⁷⁾ reported that one amylopectin cluster consists of chains with DP ≤ 24, and the chain length is closely related to the gelatinization temperature. A decrease in the numbers of chains with 6 ≤ DP ≤ 13, or an increase in the numbers of chains with 14 ≤ DP ≤ 24, leads to an increase in the gelatinization temperature. Conversely, an increase in the numbers of chains with 6 ≤ DP ≤ 13, or a decrease in the numbers of chains with 14 ≤ DP ≤ 24, leads to a decrease in the gelatinization temperature.^{18,19)} According to the criteria, the region that contributes to reducing and increasing the gelatinization temperature is shown in blue and red, respectively, in Fig. 1.

The higher gelatinization temperature in *ss1^L* was due to a reduction in the numbers of DP 7–12 short chains and an increase in the numbers of DP 16–21 chains. These changes offset the slight reduction in gelatinization temperature caused by the slight increase in the numbers of DP 6 chains (Fig. 1 (A)). The reduced gelatinization temperature in *ss3a* was due to an increase in the numbers of DP 10–12 chains and a decrease in the numbers of DP 16–19 chains, which offset the increased gelatinization temperature caused by a decrease in the numbers of DP 6–9 chains and an increase in the numbers of DP 13–15 and DP 20–24 chains (Fig. 1 (B)). The higher gelatinization temperature in *ss1^L/ss3a* was due to a decrease in the numbers of DP 7–9 chains and an increase in the numbers of DP 13–24 chains, which offset the reduction in gelatinization temperature caused by an increase in the numbers of DP 10–12 chains. The actual values of gelatinization temperature of the lines analyzed here (Table 1) agreed with the criteria described above.

Fig. 2. Typical differential scanning calorimetry (DSC) curves of purified starch from mutant lines and the wild type (one of at least three independent replications using purified starch from homogeneous plants).

Table 1. Thermal properties of purified starch in mutant lines and wild type.

	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (mJ/mg)
WT (Nipponbare)	52.9 ± 0.2 **	59.3 ± 0.1 **	65.4 ± 0.3 **	12.9 ± 0.9
<i>ss1^L</i> (<i>i2-1</i>)	54.3 ± 0.2 *	60.5 ± 0.0 **	66.1 ± 0.2 **	13.9 ± 0.6
<i>ss3a</i> (<i>e1</i>)	49.2 ± 0.4 ***	57.5 ± 0.1 ***	63.9 ± 0.2 ***	11.1 ± 0.5 ***
<i>ss1^L/ss3a</i> (#6002)	55.1 ± 0.3 *	62.5 ± 0.0 *	68.0 ± 0.0 *	13.1 ± 0.4

Data represent the mean ± SE of three replicates. Within columns, mean values of the mutant lines followed by one asterisks (*) are significantly different to the wild type according to *t*-test at $P < 0.05$. Within columns, mean values of the wild type and parent mutant lines followed by two asterisks (**) are significantly different to the double mutant line according to *t*-test at $P < 0.05$.

Fig. 3. Typical patterns of endosperm starch pasting properties measured with a rapid visco analyzer (RVA) (one of at least three independent replications using purified starch from homogeneous plants).

Pasting properties of endosperm starches.

The viscosity of gelatinized starch was measured by RVA (Fig. 3). The *ss1^L* RVA pattern was similar to that of the wild type, except that the curve was shifted so that peak viscosity temperature was slightly higher (Fig. 3). The peak viscosity of *ss3a* starch was much lower than that of the wild type⁷⁾, and the peak viscosity of *ss1^L/ss3a* was lower than that of *ss3a* (Fig. 3).

The significantly lower viscosity in *ss1^L/ss3a* endosperm starch could result from the higher amylose content [apparent amylose content was ca 1.5 times in *ss3a*⁷⁾ and *ss1^L/ss3a* of the wild type (Hayashi *et al.*, in preparation)] in *ss3a* due to the pleiotropic effects of *SSI* deficiency on *GBSSI* expression⁷⁾. However, effects of the reduced molecular mass in *ss3a* amylopectin caused by the reduction of B₂₋₃ amylopectin chains could not be excluded.^{7,20)} The reduced viscosity in *ss1^L/ss3a* endosperm starch relative to that in *ss3a* suggested that attenuation of *SSI* activity in the *ss3a* background reduced the molecular mass of amylopectin.

Morphology of endosperm amyloplasts and starch granules.

Purified starch granules were examined using SEM (Fig. 4). The size (4–5 μm) and polygonal shape of *ss1^L* starch

Fig. 4. Representative scanning electron microscopy images of purified endosperm starch granules.

Bar = 5 μm.

granules were very similar to those of the wild type. Some starch granules in *ss3a* endosperm were round-shaped and smaller (3–4 μm) than those of the wild type. Round-shaped and smaller starch granules were more abundant in *ss1^L/ss3a* than in *ss3a* (Fig. 4). The starch granules of *SSI ss1/ss3a ss3a* or *ss1ss1/SSI ss3a ss3a*, whose *SSI* or *SSI* genotypes were heterozygous, were similar to those of *ss1^L/ss3a*.⁸⁾ These results suggest that the reduction of *SSI* and the deficiency of *SSI* generate round-shaped starch granules that are smaller.

Fig. 5. (A) Representative scanning electron microscopy (SEM) images show the central regions of mature seeds from various mutant lines and wild type, (B) Amyloplast units shown in white dashed lines of SEM images.

Bar = 5 μm.

Table 2. Relative starch crystallinity (RSC) and relative amylopectin crystallinity (RAC) of purified starch.

	RSC	RAC
WT (Nipponbare)	2.69 ± 0.10 ** (100)	3.27 ± 0.12 ** (100)
<i>ss1^L</i> (<i>i2-1</i>)	2.43 ± 0.07 ** (90.4)	2.99 ± 0.09 * (91.5)
<i>ss3a</i> (<i>e1</i>)	2.36 ± 0.04 * (87.6)	3.32 ± 0.05 ** (101.6)
<i>ss1^L/ss3a</i> (#6002)	2.07 ± 0.03 * (76.9)	2.94 ± 0.04 * (89.9)

Data represent the mean ± SE of three replicates. Within columns, mean values of the mutant lines followed by one asterisks (*) are significantly different to the wild type according to *t*-test at *P* < 0.05. Within columns, mean values of the wild type and parent mutant lines followed by two asterisks (**) are significantly different to the double mutant line according to *t*-test at *P* < 0.05. The values of the parentheses are percent of the wild type.

However, the starch granule morphology in *ss1^L/ss3a* differs from that of the *ss3a/ss4b* double-mutant lines whose starch granules were almost completely round-shaped with uniform size (Toyosawa *et al.*, submitted).

Seeds were cross-sectioned, and the central regions of the sections were observed by performing SEM (Fig. 5 (A)). Rice endosperm starches were compound grains, in which several starch granules accumulated in one amyloplast.²¹⁾ Both inside and outside images of amyloplasts were obtained in the wild type and *ss1^L* (Fig. 5 (A)). Several polygonal starch granules were tightly packed in the endosperm amyloplasts of the wild type and *ss1^L*. Most of the amyloplast images were outside, and there were many gaps between amyloplasts in *ss3a* (Fig. 5 (B)). In *ss1^L/ss3a* endosperm, there were many gaps between starch granules and between amyloplasts (Fig. 5 (A)). The smaller, round-shaped starch granules in *ss1^L/ss3a* could be caused by a slightly low level of starch biosynthesis due to limited SS activity. The starch granules in shriveled seeds of phosphorylase-deficient mutant lines, in which starch biosynthesis is significantly repressed, were small and partially spherical.²²⁾

Endosperm starch granule crystallinity.

The crystallinity of starch granule in endosperm from double-mutant and parental lines was determined by measuring the X-ray diffraction of purified starch granules. The relative starch crystallinity (RSC) was calculated by adding calcium fluoride (CaF₂) as an internal standard (Table 2). The deficiency of SSIIIa and/or the reduction of SSI in *ss1^L/ss3a* led to a lower RSC than that of the wild type (Table 2). RSC of *ss1^L/ss3a* was the lowest in the lines tested, although the ΔH value by DSC of *ss1^L/ss3a* was larger than that of *ss3a* (Table 1). It might be caused by the higher frequency of the long double helices in *ss1^L/ss3a* starch than in *ss3a*. It is known that amylopectin is the main component that determines starch crystallinity.²³⁾ Therefore, the relative amylopectin crystallinity (RAC) was calculated from the apparent amylose content and RSC (Table 2). The RAC of *ss3a* was similar to that of the wild type, and the RAC of *ss1^L* and *ss1^L/ss3a* was 91.5% and 89.9% of the wild type, respectively. These results suggest that the reduction of SSI caused the slight reduction of amylopectin crystallinity.

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