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Lack of starch synthase IIIa and high expression of granule-bound starch synthase I synergistically increase the apparent amylose content in rice endosperm

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ABSTRACT

Rice endosperm starch is composed of 0–30% linear amylose, which is entirely synthesized by granule-bound starch synthase I (GBSSI: encoded by *Waxy*, *Wx*). The remainder consists of branched amylopectin and is elongated by multiple starch synthases (SS) including SSI, IIa and IIIa. Typical japonica rice lacks active SSIIa and contains a low expressing Wx^b causing a low amylose content (ca. 20%).

WAB2-3 ($SS3a/Wx^a$) lines generated by the introduction of a dominant indica Wx^a into a japonica *waxy* mutant ($SS3a/wx$) exhibit elevated GBSSI and amylose content (ca. 25%). The japonica *ss3a* mutant ($ss3a/Wx^b$) shows a high amylose content (ca. 30%), decreased long chains of amylopectin and increased GBSSI levels. To investigate the functional relationship between the *ss3a* and Wx^a genes, the $ss3a/Wx^a$ line was generated by crossing $ss3a/Wx^b$ with $SS3a/Wx^a$, and the starch properties of this line were examined. The results show that the apparent amylose content of the $ss3a/Wx^a$ line was increased (41.3%) compared to the parental lines. However, the GBSSI quantity did not increase compared to the $SS3a/Wx^a$ line. The amylopectin branch structures were similar to the $ss3a/Wx^b$ mutant. Therefore, Wx^a and *ss3a* synergistically increase the apparent amylose content in rice endosperm, and the possible reasons for this increase are discussed.

1. Introduction

Plants store large amounts of photosynthetic products as insoluble starch. This starch is initially formed as transient starch in the chloroplasts of source tissues such as leaves and stems during the day. Following degradation and translocation, the majority of the transient starch is used to form storage starch in the amyloplasts of sink tissues such as seeds and tubers [1]. The stored starch can be utilized as a carbon source for germination [2]. The stored starch in rice seeds makes up the majority of the seed weight and provides an efficient source of carbohydrates for humans all over the world [3].

Rice endosperm starch is comprised of 0–30% linear amylose [4]. The remainder is branched and highly organized clusters, which form amylopectin [5]. The ratio of amylose to amylopectin is an important factor in determining the properties of starch [6]. Starch with high amylose content has many unique characteristics such as high gelling strength, excellent film-forming ability [7,8] and ease of retrogradation [9]. These features are particularly attractive in food and non-food industrial applications; for examples, making sweets, coating for the fried food, producing adhesives, paper [7,8] and biodegradable plastics [10]. High amylose starch can also be a resistant starch which is not easily digested by humans. It provides the public with a health benefit by lowering glycemic index [11] and promoting colon health by serving as a prebiotic [12].

Extremely high amylose content (50–90%) cultivars have been identified in plants including wheat [13,14], barley [15,16], maize [17] and potato [18]. On the other hand, the maximum percentage of rice amylose content reported was 33% using the gel filtration method [5,6,19,20]. This reported percentage of total starch is lower than other analyzed crops. Therefore, possible factors for the enhancing the amylose content in rice endosperm are analyzed in this study.

Abbreviations: GBSS I, granule-bound starch synthase I; *Wx*, *Waxy*; SS, starch synthase; AGPase, ADP-glucose pyrophosphorylase; DP, degree of polymerization; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Fr, fraction; ELC, extra-long chain.

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The amylose in rice endosperm can only be synthesized by GBSSI which is tightly bound to starch granules and elongates α-1,4 linkages of glucose polymers using ADP-glucose generated by ADP-glucose pyrophosphorylase (AGPase) [21–23]. Rice mutants lacking GBSSI activity, *waxy* (*wx*), produce amylose free rice [4,24] known as glutinous rice. By contrast, the synthesis of amylopectin occurs *via* multiple isoforms of starch biosynthetic enzymes. Three isozymes of starch synthase (SSI, SSIIa and SSIIIa) are highly expressed in rice endosperm [25,26], and these isozymes elongate glucose polymers using ADP-glucose as a substrate [23]. In addition, branching enzymes, which form α-1,6 linkages, and debranching enzymes, which remove improper branches, are also necessary for the formation of amylopectin cluster structures [21,27]. Among the many SS isozymes, each SS enzyme has a distinct specificity in terms of the preferred glucan length, although some redundancy has been observed from single and/or double mutants [19,20,28,29].

In general, rice cultivars can be divided into two groups, japonica (*SS1/ss2a/SS3a/Wx^b* genotype) and indica (*SS1/SS2a/SS3a/Wx^a* genotype) rice. Typical japonica rice is oval-shaped and exhibits moderate stickiness when cooked. By contrast, typical indica rice has a more narrow and longer grain and is less sticky when cooked [30]. This textural difference results from the specific alleles of GBSSI [31,32] and SSIIa [33,34]. Japonica rice contains *Wx^b* which has a mutation at the 5′ end of intron one. This causes reduced mRNA and GBSSI protein levels, leading to reduced amylose content (ca. 20%) [24,31,35–37] as compared to indica rice (ca. 25%). Indica rice contains the highly expressed *Wx^a* allele [38]. Most japonica rice lacks active SSIIa enzyme due to two amino acid substitutions [34]. These substitutions result in reduction of short glucose chains with a degree of polymerization (DP) less than 12 in amylopectin [34,39,40].

An increase in amylose content can be accomplished by abolishing enzymes involved in amylopectin synthesis [19,20,29,41,42] or by raising the expression levels of GBSSI [19,42,43]. For example, enhanced levels of GBSSI by introduction of the *Wx^a* gene into a japonica *wx* mutant resulted in elevated amylose content (ca. 25%) as compared to *SS3a/Wx^b* (ca. 20%) or *SS3a/wx* (0%) [43]. In addition, lack of SSIIIa expression in *ss3a/Wx^b* genotype also promotes an increase in amylose content (up to ca. 30%) accompanied by an increase in the levels of both GBSS I protein and AGPase activities [19,20,41]. The elevated amylose content along with decreased amylopectin content *via* mutation of *SSIIIa* homologs has also been observed in *Chlamydomonas* [44], barley [45] and maize [46,47]. Evidence suggests that SSIII is not only involved in the elongation of long chain of amylopectin, but also in regulating the activities and expression levels of other SS isozymes. For example, SSIII absence results in an increase in SSI activities in rice [19] and maize [48]. Absence of SSIII leads to an increase in the total starch content in *Arabidopsis* [49].

Previous efforts have been directed towards understanding the function of individual SS isozymes by analyzing the unique characteristics of the starch from single and/or double mutants with *Wx^b* background [19,20]. These studies provided evidence for a relationship between *SSIIIa* and *Wx^b* gene expression [19,20]. However, rice lines lacking *SSIIIa* but containing the *Wx^a* gene were not available. Therefore, to provide insight into the relationship between *SSIIIa* and *Wx^a* and to investigate whether a new rice line will show any change in its endosperm starch properties, the *ss3a/Wx^b* mutant [19] was crossed with a WAB2-3 (*SS3a/Wx^a*) line [43,50]. After screening the offspring with *ss3a/Wx^a* genotypes, the starch properties including amylose content, amylopectin structure and the expression levels of starch synthases of the F_3 progeny were examined in this study.

2. Materials and method

2.1. Plant materials

The rice lines used in this study are as follows and their genotypes are summarized in Table 1; *Oryza sativa* L. Japonica, cv. Nipponbare (*SS3a/Wx^b*), cv. Musashimochi (*SS3a/wx*), WAB2-3 (*SS3a/Wx^a*) [43], and an *ss3a* which is a retrotransposon *Tos17* insertion mutant (*ss3a/Wx^b*) [19]. The *ss3a/Wx^b* and *SS3a/Wx^a* plants were crossed. The resulting F_1 seedlings were grown and self-pollinated to obtain F_2 progeny. The *ss3a/Wx^a* lines were screened from F_2 seedlings as described in Section 2.2. The *ss3a/Wx^a* lines were selected and a possible homozygous line was used in this study. All rice lines were grown in a closed green houses at either Akita Prefectural University or Niigata University during the summer months under natural light conditions.

2.2. Screening methods for the presence of Wx^a and absence of SSIIIa

To select the *ss3a/Wx^a* lines, the *Tos17* insertion into *SSIIIa* was PCR-screened using genomic DNA obtained from young F_2 seedlings and pairs of *Tos17* and *SSIIIa* gene-specific primers as described [19]. After confirmation of the absence of *SSIIIa*, the seedlings were grown and genomic DNA was isolated from leaf samples. The presence of the *Wx^a* transgene and the transgene copy number were analyzed using leaf genomic DNA *via* Southern blotting as described [50]. Upon confirming the presence of the *Wx^a* gene and the insertion of *Tos17* into *SSIIIa*, the selected rice plants with *ss3a/Wx^a* were further grown and the resulting seeds were used for study.

2.3. Extraction of total proteins from mature endosperm

For use in western blotting and protein quantification, total proteins were extracted. Mature rice seeds were cut in half and crushed into a powder using pliers. Then, 10 μL/mg of denaturing extraction buffer (0.125 M Tris–HCl, pH 6.8, 8 M

Table 1
Summary of SS expression levels and starch properties for the rice lines used in this study.

Line	Genotype	SS levels[a]			Amylose content[b]	Amylopectin structure[c]
		SSI	SSIIIa	GBSSI		
Nipponbare	*SS3a/Wx^b*	+	+	+	20%	Wild type
ss3a	*ss3a/Wx^b*	+	–	++	30%	Reduction in B_{2-3} chains
Musashimochi	*SS3a/wx*	+	+	–	0%	Similar pattern as Nipponbare
WAB2-3	*SS3a/Wx^a*	+	+	+++	25%	Similar pattern as Nipponbare
ss3a/Wx^a	*ss3a/Wx^a*	+	–	+++	41%	Similar pattern as *ss3a*

[a] Protein expression levels for SSI, IIIa and GBSSI were estimated by western blot as shown in Fig. 2B.
[b] Amylose contents estimated from gel filtration chromatography and their details are described in Fig. 3.
[c] The results from amylopectin structure analyses are shown in Fig. 4.