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# Characterization of the endosperm starch and the pleiotropic effects of biosynthetic enzymes on their properties in novel mutant rice lines with high resistant starch and amylose content

Yuuki Itoh, Naoko Crofts, Misato Abe, Yuko Hosaka, Naoko Fujita*

*Department of Biological Production, Akita Prefectural University, Akita 010-0195, Japan*

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ABSTRACT

Resistant starch (RS) is beneficial to human health. In order to reduce the current prevalence of diabetes and obesity, several transgenic and mutant crops containing high RS content are being developed. RS content of steamed rice with starch-branching enzyme (BE)IIb-deficient mutant endosperms is considerably high. To understand the mechanisms of RS synthesis and to increase RS content, we developed novel mutant rice lines by introducing the gene encoding starch synthase (SS)IIa and/or granule-bound starch synthase (GBSS)I from an indica rice cultivar into a japonica rice-based BEIIb-deficient mutant line, *be2b*. Introduction of *SSIIa* from an indica rice cultivar produced higher levels of amylopectin chains with degree of polymerization (DP) 11–18 than those in *be2b*; the extent of the change was slight due to the shortage of donor chains for SSIIa (DP 6–12) owing to BEIIb deficiency. The introduction of *GBSSI* from an indica rice cultivar significantly increased amylose content (by approximately 10%) in the endosperm starch. RS content of the new mutant lines was the same as or slightly higher than that of the *be2b* parent line. The relationship linking starch structure, RS content, and starch biosynthetic enzymes in the new mutant lines has also been discussed.



## 1. Introduction

Controlling the intake and type of dietary carbohydrate is important for roughly 400 million diabetic patients around the world. Resistant starch (RS) is defined as starch or starch products that resist digestion as they pass through the small intestine [1]. RS is beneficial to human health and contributes to improvement of the colonic environment. Starch is composed of essentially linear glucose polymers of amylose and frequently branched amylopectin, and the ratio of amylose to amylopectin in starch, in addition to the ratio of long to short branches within the amylopectin, influence RS content [2]. When RS values of steamed rice are less than 4%, the RS values correlate with the amylose content ($r^2 = 0.733$). In contrast, when RS values of steamed rice are ≥15%, the RS values are correlated with the ratio of amylopectin long chains rather than the apparent amylose content ($r^2 = 0.987$) [2]. To minimize the current frequency of diabetes and obesity, several transgenic and mutant crops containing high levels of RS are being developed, such as barley with mutant starch synthase (SS)IIa [3], transgenic wheat with RNA interference-mediated silencing of starch-branching enzyme (BE)IIa and BEIIb [4], rice with RNA silencing-mediated down-regulation of BEIIb [5,6], and barley with suppressed BE activity [7]. Among the mutant rice lines with starch biosynthetic enzymes on the background of japonica rice cultivars [8,9], steamed rice of BEIIb-deficient mutant lines also contains considerably higher RS than other mutant lines [2]. This strongly suggests that high levels of amylopectin long chains due to BEIIb deficiency are the most effective way to increase RS content. High amylose content resulting from high expression of *GBSSI* is also essential for high RS accumulation. In the case of barley [3,10], wheat [11], and maize [12], deficiency of SSIIa produces high amylose and RS content. By contrast, SSIIa deficiency in japonica rice cultivars does not increase amylose in the rice endosperm [13]. The effects of SSIIa and other starch biosynthetic enzymes on RS levels are still obscure in rice.

Food texture of japonica rice cultivars is different from that of indica cultivars owing to the differences in their amylopectin structure and amylose content [13,14]. *SSIIa* and granule-bound starch synthase I (*GBSSI*) gene polymorphisms in these cultivars are the

*Abbreviations:* AGPase, ADP-glucose pyrophosphorylase; SS, starch synthase; GBSS, granule-bound starch synthase; DP, degree of polymer; Fr., fraction; RS, resistant starch; MNU, N-methyl-N-nitrosourea; GM, genetically modified; SNPs, single nucleotide polymorphisms; CAPS, derived cleaved amplified polymorphic sequenced.

* Corresponding author.
*E-mail address:* naokof@akita-pu.ac.jp (N. Fujita).

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primary reason for such differences—both genes in the indica cultivar are wildtype [15–17], whereas their SNPs (single nucleotide polymorphisms) in the japonica cultivar lead to the resultant low activity of SSIIa [16,17] and GBSSI enzymes [15]. SSIIa activity in the japonica cultivar is 10% of that of the wildtype indica SSIIa due to three SNPs in the japonica *SSIIa* [16]. The lower SSIIa activity leads to a decrease in the levels of amylopectin chains with DP 13-24, and an increase in those with DP 6-12 [16,17]. Changes in amylopectin structure of japonica cultivar also lead to a lower gelatinization peak temperature than that of the indica cultivar by 5–10 °C [13]. Transcripts of japonica *GBSSI* contain a longer mRNA, including intron 1 with an incomplete splicing, in addition to a complete mRNA [18,19]. This results in reduced production of GBSSI and its activity, with the apparent amylose content of the japonica cultivar being 5%–10% lower than that of the indica cultivar [18,20].

Introduction of the indica cultivar-derived wildtype *SSIIa* into the japonica cultivar recovers the starch amylopectin structure and gelatinization peak temperature [16]. When the wildtype *SSIIa* is introduced in an *isoamylase 1* (*isa1*) mutant cultivar—which accumulates a highly soluble branched phytoglycogen in its endosperm—the chains within one cluster of amylopectin (DP ≤ 24) are elongated, with three longer glucose residues, and certain α-glucans are turned insoluble [21]. Amylose content of a transgenic rice line, produced by introducing *GBSSI* from the indica cultivar in a glutinous (*gbss1* or *waxy* mutant) rice line (japonica cultivar), was comparable to that of the indica cultivar [22]. An SSIIIa-deficient mutant rice line with high amylose content (i.e., apparent amylose content of around 30%) crossed with the transgenic line expressing indica type *GBSSI* showed an even higher apparent amylose content of around 40%, which is also more than that of the parental lines [23].

In the present study, we developed novel mutant rice lines by introducing *SSIIa* and *GBSSI* from the indica cultivar into a BEIIb-deficient mutant line, *be2b*, in order to develop rice lines with high quantity of RS by using a non-genetic modification method such as crossing [24]. Endosperm starch content of these lines and that of parental lines was compared, and the relationship linking starch structure, RS content, and starch biosynthetic enzymes in the new mutant lines was discussed.

## 2. Materials and methods

### 2.1. Plant materials

To introduce indica *SSIIa* and *GBSSI* (denoted hereon as $SSIIa^I$ and $GBSSI^I$, respectively) into the BEIIb-deficient *be2b, EM10* (screened from the MNU [N-methyl-N-nitrosourea] mutagenesis of Kinmaze (japonica cultivar, [25,26]); *EM10* was crossed with Kasalath (indica cultivar) (Fig. 1). The resulting double heterozygotes ($F_1$) were self-pollinated. Total protein was extracted from one-fourth sections of mature opaque seeds—which is a characteristic trait attributable to *be2b*—from $F_2$ populations, and immunoblotting was performed using rice BEIIb anti-serum [26]. The remaining three-fourth portions of mature seeds with embryos deficient in BEIIb were grown, and genomic DNA was extracted from young leaves. The new mutant lines, with genotypes $SSIIa^J SSIIa^J/GBSSI^J GBSSI^J/be2bbe2b$ (#1203B; $SSIIa^J$ derived from japonica cultivars, Kinmaze) and $SSIIa^I SSIIa^I/GBSSI^I GBSSI^I/be2bbe2b$ (#1203C), were selected as described [24] by derived cleaved amplified polymorphic sequence (dCAPS) markers (Fig. 2A) and PCR (Fig. 2B, C). The dCAPS primers for selecting SSIIa genotypes included a forward primer with a *Xho*I site (5′-CCAACTACACCGTGGCCTCGCTGGACTCG-3′) and a reverse primer (5′-GCCGATGATGTCCACACCTT-3′). After amplification, PCR products were digested with *Xho*I. $SSIIa^I$ produced a 149-bp band, and $SSIIa^J$ produced 119-bp and 30-bp bands (Fig. 2A). Two mutant lines of #1203B (#1203B21 and #1203B23) and #1203C (#1203C11 and #1203C32) were isolated, and $F_3$ and $F_4$ seeds of these lines were analyzed. Kinmaze (the parental line of *EM10*; japonica rice cultivar) and Kasalath (indica rice cultivar) were used as controls, and *EM10* was also used as the parental line (Table 1). The plants were grown during summer in an experimental paddy field at the Akita Prefectural University under natural environmental conditions, except for the #1203B lines, the seeds of which were grown in a green house after flowering because the flowering time is delayed for these lines.

**Table 1**
Genotypes and dehulled-grain weights of mutant and parental rice lines.

| Line | Genotype | Seed weight (mg) | |
|---|---|---|---|
| Kasalath | $SSIIa^I/GBSSI^I/BEIIb$ | 15.4 ± 0.2 [b] | (81)[d] |
| Kinmaze | $SSIIa^J/GBSSI^J/BEIIb$ | 19.0 ± 0.3 [a] | (100) |
| *EM10* | $SSIIa^J/GBSSI^J/be2b$ | 10.7 ± 0.3 [c] | (56) |
| #1203B21 | $SSIIa^J/GBSSI^J/be2b$ | 15.1 ± 0.4 [b] | (80) |
| #1203B23 | $SSIIa^J/GBSSI^J/be2b$ | 15.5 ± 0.2 [b] | (81) |
| #1203C11 | $SSIIa^I/GBSSI^I/be2b$ | 15.1 ± 0.2 [b] | (79) |
| #1203C32 | $SSIIa^I/GBSSI^I/be2b$ | 15.7 ± 0.3 [b] | (82) |

Dehulled-grain weight was estimated as mean ± SE using 20 individual mature seeds per line.
Samples annotated with different letters (a–c) are significantly different from one another as determined using the Tukey-Kramer method ($p < 0.05$).
[d] Percentage of Kinmaze.

### 2.2. Extraction of protein fractions and immunoblotting

Soluble protein (SP), loosely bound starch granule protein (LBP), and tightly bound starch granule protein (TBP) were extracted from developing and mature endosperms in accordance with a previously published method [27]. Immunoblotting was performed as previously described [23].

### 2.3. Enzyme assay

Samples used for Native-PAGE/activity staining were extracted using equal volumes of buffer to fresh weight of seeds, and the same quantities were loaded among the different lines as described [28]. SS activity staining was performed as described previously [26], with a slight modification of adding 0.5 M citrate in the reaction mixture. BE activity assessment were performed using gels containing 0.0001% oyster glycogen [29]. Debranching enzyme (DBE) assessment was performed as described [28]. ADP-glucose pyrophosphorylase (AGPase) activity was assayed according to [30].

### 2.4. Analyses of starch

Starch granules were purified from polished mature rice grains using the methods [31,32]. Chain-length distribution of endosperm starch and gel filtration were performed according to the method described [27]. Thermal properties of endosperm starch were assessed by differential scanning calorimetry (DSC) in accordance with a previously published method [33].

### 2.5. Measurement of RS levels

RS levels in steamed rice were estimated using an RS assay kit (Megazyme, Ireland) according to the method [2], but with a slight modification—1.8 times of the recommended volume of water was used to steam the polished rice grains.