Note

Screening Method for Novel Rice Starch Mutant Lines Prepared by Introducing Gene Encoding Starch Synthase IIa and Granule-bound Starch Synthase I from Indica Cultivar into a Branching Enzyme IIb-Deficient Mutant Line

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Abstract: The structure and properties of starch reserves in rice seeds are strongly affected by deficiencies in specific starch biosynthetic enzymes, which are highly expressed in storage tissues. Rice lines with unique seed starches should be utilized for food and industrial applications in the near future. We are currently developing novel rice mutant lines with distinct starch properties by introducing specific genes from different cultivars into mutant lines and by producing multiple combinations of single mutant lines. Obtaining the homozygous genotypes of the target genes is necessary during the screening process of these materials. In this study, we developed an effective, accurate screening method for identifying rice lines with novel starch composition. Specifically, we produced a novel mutant line in which we introduced genes encoding starch synthase IIa (SSIIa) and granule-bound starch synthase I (GBSSI) from indica cultivar into a starch branching enzyme IIb (BEIIb)-deficient mutant line.

Key words: novel starch, rice mutant, screening method, starch branching enzyme, starch synthase

Rice is a staple food for over half the world’s population.1) The standard rice consumed in Japan is steamed rice from japonica cultivars. Numerous good-tasting japonica cultivars with excellent agricultural traits have been developed at agricultural stations. The starch properties in japonica rice cultivars are quite different from those of indica rice cultivars. These differences are mainly explained by the single nucleotide polymorphisms (SNPs) in the starch synthase IIa and granule-bound starch synthase I genes (SSIIa and GBSSI, respectively) in japonica cultivars, whereas indica cultivars are thought to have wild-type alleles of these genes.2,3) Glutinous rice cultivars are gbs1 null mutant lines containing amylase-free starch. Thus, spontaneous mutant lines with distinct starch traits in the endosperm have long been used for human consumption.

Since 2000, numerous rice starch mutant lines have been generated to elucidate the functions of starch biosynthetic enzymes. Among these, the starch properties in the endosperm of SSI, SSIIa, SSIIa, GBSSI, BEI, BEIIb, and ISAI-deficient mutant lines are quite different from those of the wild type.4,5) Double mutant lines representing different combinations of these single mutant lines are also different from the wild type and their parental mutant lines.6–10) These mutant lines are in the japonica background (Nipponbare, Taiichung 65, or Kimmaze), suggesting that the introduction of SSIIa and GBSSI from indica cultivars into these mutant lines would increase the diversity of starch properties.

When screening for such mutants, obtaining the homozygous genotypes of the target genes is indispensable. Here, we describe an effective, accurate method for screening novel starch rice lines, for example, a novel mutant line containing SSIIa and GBSSI genes introduced from indica cultivars into a starch branching enzyme IIb (BEIIb)-deficient mutant line (SSIIa/GBSSI/be2b).

The novel mutant line, SSIIa/GBSSI/be2b, was generated by crossing a BEIIb-deficient mutant (EM10, SSIIa/GBSSI/be2b)11) with an indica rice cultivar, Kasalath (SSIIa/GBSSI/BEIIb). GBSSI (corresponding to Wx) represents wild-type GBSSI derived from indica cultivar, Kasalath, in this study, and is highly expressed in developing endosperm.12) On the other hand, GBSSI (corresponding to Wx) represents leaky gbs1 mutant with low expression levels, which was derived from japonica cultivar, Kinmaze, in this study. SSIIa from japonica cultivars is almost inactive, whereas SSIIa from indica cultivars is active.13) After screening, the rice plants were grown during the summer of 2014 in an experimental paddy field at Akita Prefectural University under natural environmental conditions.

Total protein was extracted from 1/4 sections of mature rice endosperm and immunoblotting was performed to screen F2 seeds using rice BEIIb anti-serum11) as described in Crofts et al.14) Extraction of soluble, loosely-bound and tightly-bound starch granules proteins from mature F2 seeds and subsequent immunoblotting were performed as previously described.15,16) A volume of 5 µL per sample was

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2) Abbreviations: BE, starch branching enzyme; DP, degree of polymerization; GBSSI, granule-bound starch synthase I; PCR, polymerase chain reaction; RS, resistant starch; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SS, starch synthase.
used for western blotting. The dilution factors of primary antibodies used in this study were as follows: anti-SSI 1:1,000, anti-SSIIa 1:1,000, anti-BEIIb, and anti-GBSSI 1:1,000.

Screening for the active SSIIa genotype was performed using SNV-Specific PCR primers as previously described. Screening for the GBSSI genotype was also performed by PCR using SNV-specific PCR primers as previously described. The primer sequences were as follows: SSIIa, 5′-GGCTAC TAAA-3′; SSIIa, 5′-GGCTAC TAAA-3′; 5′-CGGGCTGAGGGACATCG-3′; SSIIa, 5′-CAGGAAAGAACATCGCAAGG-3′ and 5′-TGACCAACTCTGGCTACTAAA-3′; GBSSI, 5′-CAGGAAAGAACATCGCAAGG-3′ and 5′-TGACCAACTCTGGCTACTAAA-3′. The PCR conditions for SSIIa and GBSSI were as follows: 94 °C for 2 min, and 38 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 30 sec using Quick Taq HS dye mix (TOYOBO Co., Ltd., Osaka, Japan). All reactions were performed in a volume of 10 μL.

The rice be2b mutant line (EM10) accumulates starch with ultrahigh resistance to gelatinization, with amylepectin containing significantly low levels of short chains (DP ≤ 13) and high levels of long chains (DP > 14) in the endosperm. BEIIb-deficient mutant lines have the highest levels of resistant starch among several high amylese rice lines (Tsuki et al., submitted). High amylese content and/or amylepectin with high levels of long chains (DP ≥ 30) are required for high RS content rice (Tsuki et al., submitted). Since the apparent amylese content of be2b is not particularly high (ca. 28 %), this mutant is expected to have a potentially higher RS content by increasing the amylese content. Thus, we tried to develop the novel mutant line, SSIIa/GBSSI/be2bbe2b (#1203C), by introducing SSIIa and GBSSI from indica cultivar into the be2b mutant background (EM10) in order to gain active SSIIa and high expression level of GBSSI.

The scheme used for screening of #1203C is shown in Fig. 1. In this study, we introduced SSIIa and GBSSI from an indica rice cultivar, ‘Kasalath’ using traditional crossing methods because it is difficult to used transgenic plants for food applications. First, the be2b mutant line of EM10 was crossed with indica cultivar Kasalath. A total of 1,413 F2 seeds were obtained by self-pollination of five F1 seeds. The seed morphology of the BEIIb-deficient mutant, EM10, is opaque, whereas that of the wild type is translucent (Fig. 1). Therefore, opaque seeds, suggesting the presence of the be2bbe2bbe2b endosperm genotype, were collected from the F2 seed population. A total of 106 opaque seeds were obtained from the F2 seed population (Fig. 1; Table 1). Secondly, total protein was extracted from 1/4 portions of non-embryo side of each opaque seed, and the deficiency in BEIIb protein was confirmed by immunoblotting using anti-BEIIb serum (Figs 1 and 2). In order to obtain more homozygous plants with SSIIa/SSIIa/GBSSI/GBSSI/be2bbe2b genotype, we also screened F2 seeds (#1206) of cross between #1203 (EM10 x Kasalath), and selected SSIIa/SSIIa/SSIIa/SSIIa/GBSSI/GBSSI/be2bbe2b genotype plants. 82 BEIIb-deficient seeds by immunoblotting screening from F2 seeds of #1203 and #4019 (ss3a/be2b) and Kasalath as well as #1203 (EM10 x Kasalath), and selected SSIIa/SSIIa/SSIIa/SSIIa/GBSSI/GBSSI/be2bbe2b genotype plants. 82 BEIIb-deficient seeds by immunoblotting screening from F2 seeds of #1203 and #4019 (ss3a/be2b) and Kasalath as well as #1203 (EM10 x Kasalath), and selected SSIIa/SSIIa/SSIIa/SSIIa/GBSSI/GBSSI/be2bbe2b genotype plants. 82 BEIIb-deficient seeds by immunoblotting screening from F2 seeds of #1203 and #4019 (ss3a/be2b) and Kasalath as well as #1203 (EM10 x Kasalath), and selected SSIIa/SSIIa/SSIIa/SSIIa/GBSSI/GBSSI/be2bbe2b genotype plants. 82 BEIIb-deficient seeds by immunoblotting screening from F2 seeds of #1203 and

Table 1. Number of seeds identified by screening.

| Stage of screening | Number of seeds |
|--------------------|-----------------|
| F2 seeds           | 1413 (100)      |
| Opaque seeds       | 106 (7.5)       |
| BEIIb-deficient seeds by immunoblotting screening | 82 (5.8) |
| SSIIa/SSIIa/GBSSI/GBSSI/be2bbe2b by PCR screening | 23 |

Number of seeds from five F2 rice plants. % of F2 seeds. Total numbers from #1203 F2 seeds and #1206 F2 seeds, which is cross between #4019 (ss3a/be2b) and Kasalath.
#1206 were obtained (Table 1). The remaining portion of each confirmed BEIIb-deficient seed (including the embryo) was transplanted to agar medium in a Petri dish to obtain as many healthy plants as possible while avoiding contamination. After approximately seven days, germinated plants were transplanted to a cell tray (Fig. 1). Thirdly, extracted genomic DNA from young leaves was used for PCR screening with primer pairs for SSIIa and GBSSI including SNPs (Figs 1 and 3). If the plant had a SSIIa/SSIIa' homozygous genotype, a PCR band would be detected when the SSIIa' primer set was used, whereas a PCR band would not be detected when the SSIIa' primer set was used. If the plant had a SSIIa'/SSIIa' homozygous genotype, SSIIa' primer set would produce faint bands, whereas a PCR band would be detected when the SSIIa' primer set was used. If the plant had a GBSSII/GBSSII' homozygous genotype, a PCR band would be detected when the GBSSII' primer set was used, whereas if the plant had a GBSSII'/GBSSII' homozygous genotype, a PCR band would be detected when the GBSSII' primer set was used (Fig. 3).

Among the PCR screening of survived 72 young plants DNA, 30 SSIIa/SSIIa' homozygous, 13 SSIIa'/SSIIa' homozygous and 29 SSIIa'/SSIIa' heterozygous, and 44 GBSSII/GBSSII' homozygous, 6 GBSSII'/GBSSII' homozygous and 22 GBSSII'/GBSSII' heterozygous plants were obtained. Finally, 23 plants with the genotype SSIIa'/SSIIa'/GBSSII/GBSSII' were obtained (Table 1). These rice plants were transplanted to the paddy field to obtain F3 seeds (Fig. 1). In rice, SSIIa' and GBSSI' are located on chromosome 6, and both genes are closely linked (http://agri-trait.dna.affrc.go.jp/). Therefore, rice plants with the SSIIa'/SSIIa'/GBSSII/GBSSII' genotype would be isolated at relatively high frequency. However, those with SSIIa'/SSIIa'/GBSSII'/GBSSII' or SSIIa'/SSIIa'/GBSSII'/GBSSII' genotypes would be isolated at low frequency (only 1 and 5 plants were obtained from the PCR screening, respectively) as recombination would have to occur between SSIIa' and GBSSI'.

To confirm the genotypes of F3 seeds, we conducted immunoblotting of proteins extracted from F3 endosperm (Fig. 4). BEIIb protein was detected from soluble and loosely granule-bound proteins from the four independent F3 seeds (Fig. 4A), and SSIIa and GBSSI' were detected from tightly granule-bound proteins (Fig. 4B). Four independent F3 seeds of #1203C lacked BEIIb protein, indicating that F3 rice plants from #1203C screened by seed morphology and immunoblotting had a homozygous be2bbe2b genotype at a frequency of 1-(1/4)^4.

SSIIa derived from indica rice cultivars is tightly bound to starch granules, whereas SSIIa' from japonica rice cultivars is not. SSIIa bands from four independent F3 seeds from #1203C were detected in the fraction of tightly granule-bound protein, whereas this band was not detected in the fraction from japonica cultivar Kinmaze. These results suggest that SSIIa' gene from Kasalath was introduced into #1203C F3 plants as homozygous. On the other hand, the
faint SSIIa band was detected from EM10 (Fig. 4B) because BEIIb-deficiency affects the degree of granule binding of several other starch biosynthetic enzymes. GBSSI protein bands from indica rice cultivars are much stronger than those from japonica cultivars. The GBSSI bands from four independent F1 seeds from #1203C were much more and denser than those from Kinmaze and EM10, indicating that GBSSII from Kasalath was introduced into #1203C F1 plants as homozygous.

These results suggest that F1 rice plants screened by the method developed in this study had a fixed genotype, SSIIa/SSIIa/GBSSII/GBSSII/be2bbe2b, and that it is possible to analyze the starch properties of F1 seeds produced from self-pollination of F1 rice plants.

The advantages of the screening method developed in this study are that this method makes it possible to begin selecting F2 seeds based on morphology and immunoblotting analysis, and to transplant seeds directly to generate F3 seeds available. As shown in this study, it is possible to perform self-pollination of F2 rice plants.

These results suggest that F2 rice plants screened by the method developed in the present study can be used to screen double recessive fixed genotype rice plants obtained from F2 seeds after backcrossing, making it possible to effectively identify novel starch mutants and cultivars with potential food and industrial applications.

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