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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 *gbss1* null mutant lines containing amylose-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, SSIIIa, 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, Taichung 65, or Kinmaze), 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 ($SSIIa^{I}/GBSSI^{I}/be2b$).

The novel mutant line, $SSIIa^{I}/GBSSI^{I}/be2b$, was generated by crossing a BEIIb-deficient mutant (*EM10*, $SSIIa^{J}/GBSSI^{J}/be2b$)[11)] with an indica rice cultivar, Kasalath ($SSIIa^{I}/GBSSI^{I}/BEIIb$). $GBSSI^{I}$ (corresponding to Wx^{a}) represents wild-type *GBSSI* derived from indica cultivar, Kasalath, in this study, and is highly expressed in developing endosperm.[12)] On the other hand, $GBSSI^{J}$ (corresponding to Wx^{b}) represents leaky *gbss1* mutant with low expression levels, which was derived from japonica cultivar, Kinmaze, in this study.[3)] $SSIIa^{J}$ from japonica cultivars is almost inactive, whereas $SSIIa^{I}$ 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 F_2 seeds using rice BEIIb anti-serum[11)] as described in Crofts *et al*.[14)] Extraction of soluble, loosely-bound and tightly-bound starch granules proteins from mature F_3 seeds and subsequent immunoblotting were performed as previously described.[15,16)] A volume of 5 μL per sample was

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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,[15] anti-SSIIa 1:1,000,[16] anti-BEIIb,[11] and anti-GBSSI 1:1,000.[15]

Screening for the active *SSIIa*^{I} genotype was performed using SNP-specific PCR primers as previously described.[17] Screening for the *GBSSI*^{I} genotype was also performed by PCR using SNP-specific PCR primers as previously described.[18] The primer sequences were as follows: *SSIIa*^{I}, 5′-CGGGCTGAGGGACATCG-3′ and 5′-ACACAAACC-GGAAGCTAAT-3′; *SSIIa*^{J}, 5′-CGGGCTGAGGGACAT-CA-3′ and 5′-CACACAAACCGGAAGCTAAT-3′; *GBSSI*^{I}, 5′-CAGGAAGAACATCTGCAAGG-3′and5′-TGACCAACTC-GGCTACTAAA-3′; *GBSSI*^{J}, 5′-CAGGAAGAACATCTG-CAAGT-3′ and 5′-TGACCAACTCGGCTACTAAA-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 amylopectin containing significantly low levels of short chains (DP ≤ 13) and high levels of long chains (DP > 14) in the endosperm.[11] BEIIb-deficient mutant lines have the highest levels of resistant starch[19] among several high amylose rice lines (Tsuiki *et al.*, submitted). High amylose content and/or amylopectin with high levels of long chains (DP ≥ 30) are required for high RS content rice (Tsuiki *et al.*, submitted). Since the apparent amylose content of *be2b* is not particularly high (ca. 28 %), this mutant is expected to have a potentially higher RS content by increasing the amylose content. Thus, we tried to develop the novel mutant line, *SSIIa*^{I}*SSIIa*^{I}/*GBSSI*^{I}*GBSSI*^{I}/*be2bbe2b* (#1203C), by introducing *SSIIa*^{I} and *GBSSI*^{I} 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*^{I} and *GBSSI*^{I} 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 *EM10* was crossed with indica cultivar Kasalath. A total of 1,413 F_2 seeds were obtained by self-pollination of five F_1 seeds. The seed morphology of the BEIIb-deficient mutant, *EM10*, is opaque, whereas that of the wild type is translucent (Fig. 1).[11,20] Therefore, opaque seeds, suggesting the presence of the *be2bbe2bbe2b* endosperm genotype, were collected from the F_2 seed population. A total of 106 opaque seeds were obtained from the F_2 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*^{I}*SSIIa*^{I} /*GBSSI*^{I}*GBSSI*^{I}/*be2bbe2b* genotype, we also screened F_2 seeds (#1206) of cross between #4019 (*ss3a/be2b*) and Kasalath as well as #1203 (*EM10* x Kasalath), and selected *SSIIa*^{I}*SSIIa*^{I}/*SSIIIaSSIIIa*/*GBSSI*^{I}*GBSSI*^{I}/*be2bbe2b* genotype plants. 82 BEIIb-deficient seeds by immunoblotting screening from F_2 seeds of #1203 and

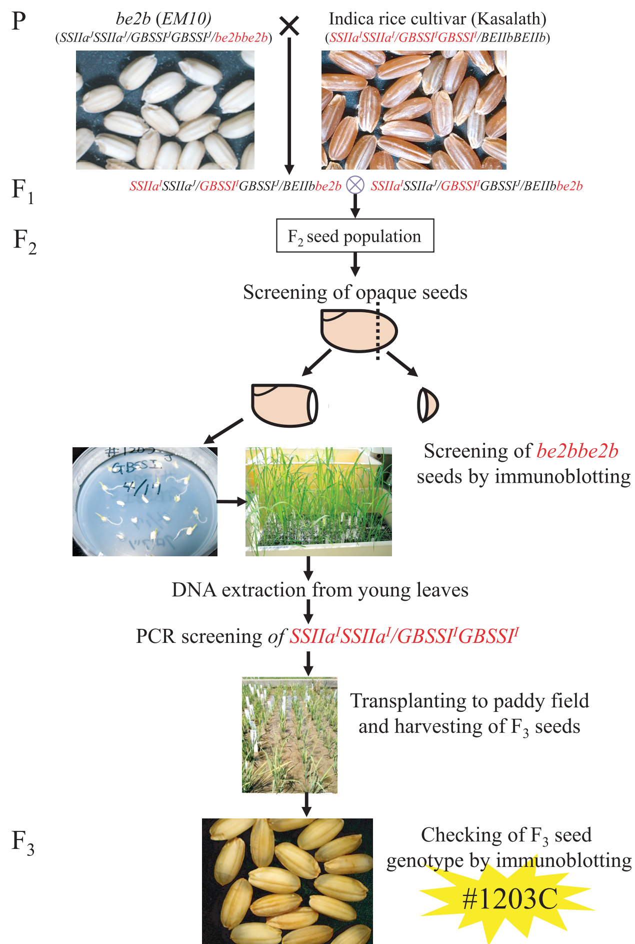


Fig. 1. Scheme of the screening of *SSIIa*^{I}*SSIIa*^{I}/*GBSSI*^{I}*GBSSI*^{I}/*be2bbe2b* rice mutant.

Table 1. Number of seeds identified by screening.

Stage of screening	Number of seeds
F_2 seeds	1413[a] (100)[b]
Opaque seeds	106 (7.5)
BEIIb-deficient seeds by immunoblotting screening	82 (5.8)
SSIIa^{I}*SSIIa*^{I}/*GBSSI*^{I}*GBSSI*^{I}/*be2bbe2b* by PCR screening	23[c]

[a]Number of seeds from five F_1 rice plants. [b]% of F_2 seeds. [c]Total numbers from #1203 F_2 seeds and #1206 F_2 seeds, which is cross between #4019 (*ss3a/be2b*)[9] and Kasalath.

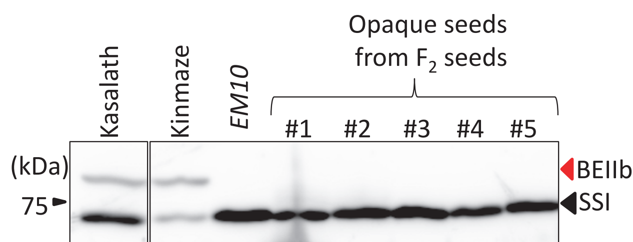


Fig. 2. Screening for BEIIb-deficiency by immunoblotting of total protein extracted from 1/4 endosperm portions of opaque F_2 seeds using anti-BEIIb serum.

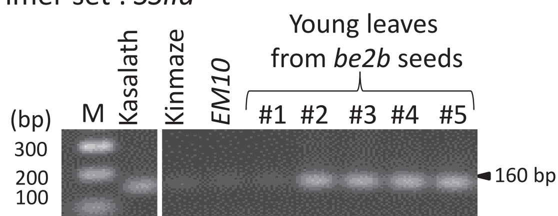
Kasalath, indica cultivars; Kinmaze, japonica cultivars, and the parent line of *EM10*; *EM10*, a BEIIb-deficient mutant. #1−5 are from five independent opaque seeds from F_2 of *EM10* × Kasalath crossing. SSI bands using anti-SSI serum were used as a control.

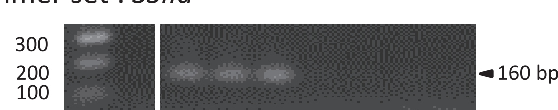
#1206 were obtained (Table 1). The remaining portion of each confirmed BEIIb-deficient seed (including the embryo) was transplanted to agar medium in a Petri dish to obtain as many healthy plants as possible while avoiding contamination. After approximately seven days, germinated plants were transplanted 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 $SSIIa^{I}SSIIa^{I}$ homozygous genotype, a PCR band would be detected when the $SSIIa^{I}$ primer set was used, whereas a PCR band would not be detected when the $SSIIa^{J}$ primer set was used. If the plant had a $SSIIa^{J}SSIIa^{J}$ homozygous genotype, $SSIIa^{I}$ primer set would produce faint bands, whereas a PCR band would be detected when the $SSIIa^{J}$ primer set was used. If the plant had a $GBSSI^{I}GBSSI^{I}$ homozygous genotype, a PCR band would be detected when the $GBSSI^{I}$ primer set was used, whereas if the plant had a $GBSSI^{J}GBSSI^{J}$ homozygous genotype, a PCR band would be detected when the $GBSSI^{J}$ primer set was used (Fig. 3).

Among the PCR screening of survived 72 young plants DNA, 30 $SSIIa^{I}SSIIa^{I}$ homozygous, 13 $SSIIa^{J}SSIIa^{J}$ homozygous and 29 $SSIIa^{I}SSIIa^{J}$ heterozygous, and 44 $GBSSI^{I}GBSSI^{I}$ homozygous, 6 $GBSSI^{J}GBSSI^{J}$ homozygous and 22 $GBSSI^{I}GBSSI^{J}$ heterozygous plants were obtained. Finally, 23 plants with the genotype $SSIIa^{I}SSIIa^{I}/GBSSI^{I}GBSSI^{I}/$ *be2bbe2b* were obtained (Table 1). These rice plants were transplanted to the paddy field to obtain F_3 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 $SSIIa^{I}SSIIa^{I}/GBSSI^{I}GBSSI^{I}$ genotype would be isolated at relatively high frequency. However, those with $SSIIa^{I}SSIIa^{I}/GBSSI^{J}GBSSI^{J}$ or $SSIIa^{J}SSIIa^{J}/GBSSI^{I}GBSSI^{I}$ 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 F_3 seeds, we conducted immunoblotting of proteins extracted from F_3 endosperm (Fig. 4). BEIIb protein was detected from soluble and loosely granule-bound proteins from the four independent F_3 seeds (Fig. 4A), and SSIIa and GBSSI were detected from tightly granule-bound proteins (Fig. 4B). Four independent F_3 seeds of #1203C lacked BEIIb protein, indicating that F_2 rice plants from #1203C screened by seed morphology and immunoblotting had a homozygous *be2bbe2b* genotype at a frequency of $1\text{-}(1/4)^4$.

SSIIa derived from indica rice cultivars is tightly bound to starch granules, whereas SSIIa from japonica rice cultivars is not.[13] SSIIa bands from four independent F_3 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 $SSIIa^{I}$ gene from Kasalath was introduced into #1203C F_2 plants as homozygous. On the other hand, the





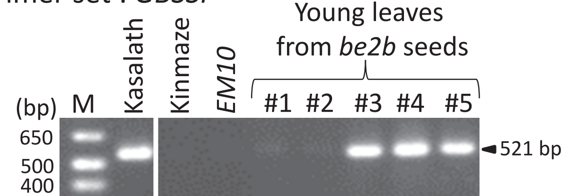


Fig. 3. Example for the PCR Screening of F_2 BEIIb-deficient seeds. A and B, PCR amplified band pattern of *SSIIa* gene using $SSIIa^{I}$ primer set (A) and $SSIIa^{J}$ primer set (B), respectively; C and D, PCR amplified band pattern of *GBSSI* gene using $GBSSI^{I}$ primer set (C) and $GBSSI^{J}$ primer set (D), respectively.

From the results, the genotypes of #1–5 were determined as $SSIIa^{J}SSIIa^{J}/GBSSI^{J}GBSSI^{J}$ (#1), $SSIIa^{I}SSIIa^{I}/GBSSI^{J}GBSSI^{J}$ (#2), $SSIIa^{I}SSIIa^{I}/GBSSI^{I}GBSSI^{I}$ (#3–5), respectively.

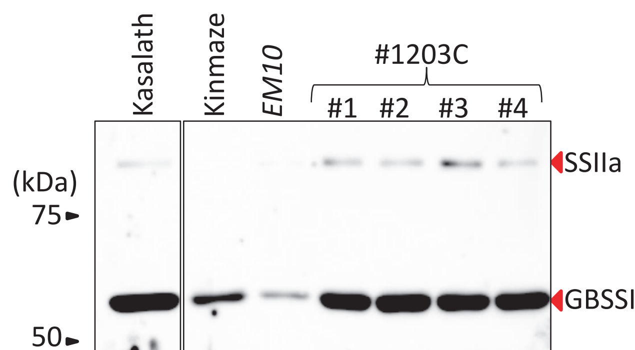


Fig. 4. Immunoblotting of F_3 seeds from #1203C and the parental lines. A. Detection of BEIIb bands of soluble and loosely-bound to starch granule proteins extracted from F_3 seeds using anti-BEIIb serum. B. Detection of SSIIa and GBSSI bands of tighly-bound proteins extracted from F3 seeds using anti-SSIIa and anti-GBSSI serum.

#1–4 are from four independent opaque seeds from F_3 seeds from #1203C. SSI bands using anti-SSI serum were used as control. Kasalath, indica cultivar; Kinmaze, japonica cultivar and the parent line of *EM10*; *EM10*, a BEIIb-deficient mutant.

faint SSIIa band was detected from *EM10* (Fig. 4B) because BEIIb-deficiency affects the degree of granule binding of several other starch biosynthetic enzymes.[9] GBSSI protein bands from indica rice cultivars are much stronger than those from japonica cultivars.[12] The GBSSI bands from four independent F_3 seeds from #1203C were much more and denser than those from Kinmaze and *EM10*, indicating that $GBSSI^I$ from Kasalath was introduced into #1203C F_2 plants as homozygous.

These results suggest that F_2 rice plants screened by the method developed in this study had a fixed genotype, $SSIIa^{I}SSIIa^{I}/GBSSI^{I}GBSSI^{I}/be2bbe2b$, and that it is possible to analyze the starch properties of F_3 seeds produced from self-pollination of F_2 rice plants.

The advantages of the screening method developed in this study are that this method makes it possible to begin selecting F_2 seeds based on morphology and immunoblotting analysis, and to transplant seeds directly to generate F_3 seeds by transplanting 3/4 sections of seeds (including embryos) that had been screened. This technique is possible because the starch property phenotypes are visible in the endosperm. Definitive selection by immunoblotting is possible if the mutation is caused by protein deficiency and anti-serum is available. As shown in this study, it is possible to perform efficient selection using a relatively small number of F_2 seeds using a trait such as opaque seed morphology caused by *be2b*. When screening for a trait that does not alter seed morphology, such as a reduction in protein activity rather than a protein deficiency (such as that of *be2a*; *EM19*),[21] large numbers of F_2 seeds must be planted for the DNA screening from young leaves using molecular markers.

The screening method developed in this study cannot only be used to isolate novel mutant lines, but it can also be used to isolate new cultivars with improved agricultural traits through backcrossing. We developed new rice cultivars by backcrossing unique starch mutant lines with elite cultivars. BC_2F_2 seeds (F_2 seeds from two rounds of backcrossing) of a double recessive mutant backcrossed with Akita 63, an ultrahigh yield rice cultivar, have a high seed weight.[4] The method developed in the present study can be used to screen double recessive fixed genotype rice plants obtained from F_2 seeds after backcrossing, making it possible to effectively identify novel starch mutants and cultivars with potential food and industrial applications.

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