Characterization and Utilization of Spontaneous Deficiency in Starch Branching Enzyme I of Rice (Oryza sativa L.)

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Abstract: Mutations in the starch synthesizing genes of cereal crops with altered starch properties has been utilized to widen the applications of grain, flour and starch. Here, rice spontaneous mutants that lacked starch branching enzyme I (BEI) activity were identified from among local upland rice cultivars. Two cultivars, Kurnai and Hiderishirazu-D, lacked BEI activity in the developing endosperm, and their reserved starch was rich in short chains of amylopectin and showed a low pasting temperature (PT) of rice flour. These features are similar to those of the induced starch branching enzyme I gene (Sbe1) mutant of rice. We detected polymorphisms in the Sbe1 of the Kurnai and Hiderishirazu-D cultivars and used them to develop PCR markers suitable for selecting breeding lines and cultivars with BEI deficiency. We assessed the possible utilization of BEI deficiency in waxy rice processing with the use of both the spontaneous and induced BEI-deficient lines. Rice cakes made from BEI-deficient lines maintained their softness for longer periods than those made from functional BEI lines. Our results suggest that use of BEI-deficient waxy rice could improve the quality and extend the shelf life of waxy rice products.

Key words: rice (Oryza sativa L.), starch branching enzyme I, amylopectin, gelatinization properties, rice cake, spontaneous mutant

Starch, the major component of cereal grains including rice is composed of two types of α-polyglucan, amylose and amylopectin. Amylose forms a mainly linear structure, with glucose residues linked via α-1,4 bonds, whereas amylopectin has a highly ordered structure with numerous branches linked to the middle of the linear structure by α-1,6 bonds. The ratio of amylose to amylopectin and the characteristics of the distribution of amylopectin chain-lengths affect the eating qualities and processing properties of cereal-based foods through changes in gelatinization and retrogradation of starch. Natural and induced mutants that have a defect in a gene encoding a starch-synthesizing enzyme and altered starch properties have been utilized in food production. For example, traditionally cultivated waxy rice, waxy maize, waxy barley, and lately bred waxy wheat, which lack the granule-bound starch synthase I (GBSSI or Waxy) responsible for amylose synthesis have stickier or softer textures after cooking, and have been utilized for food products where such textures are desirable. In case of the rice mutants lacking starch branching enzyme IIb activity (amylose-extender or ae), the proportion of short-chains (degree of polymerisation (DP) 17 or less) of amylopectin is decreased and that of middle-length chains (DP 18 to 36) is increased, which results in higher gelatinization temperatures (GTs) and faster retrogradation of gelatinized starch. This type of maize is known as amyloamaize and is utilized for the production of resistant starch: i.e., starch that cannot be digested by the human small intestine and therefore acts as a dietary fiber with many health benefits. Lack of starch synthase IIa (SSIIa) function causes changes in chain-length distribution: in rice, barley, wheat and maize, there is an

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Abbreviations: BE, starch branching enzyme; cP, centipoise; GBSS, granule-bound starch synthase; GT, gelatinization temperature; PAGE, polyacrylamide gel electrophoresis; Pho, starch phosphorylase; PT, pasting temperature; RVA, Rapid Visco Analyser; Sbe1, starch branching enzyme I gene; SNP, single nucleotide polymorphism; SS, starch synthase; UTR, untranslated region; wt, waxy; bp, base pair; In/Del, insertion/deletion.
increased proportion of short-chains (DP of around 7–10) and a decreased proportion of middle-length chains (DP of around 12–20), without obvious effects on the proportion of longer chains. In case of rice, the majority of subpecies *japonica* has amylopectin with high proportion of short chains (S-type amylopectin) while the mainstream of subspces *indica* has amylopectin with high proportion of middle-length chains (L-type amylopectin) based on the differences in SSIIa function. GTS are lower in SSIIa-deficient S-type amylopectin cultivars than those in L-type amylopectin cultivars with functional SSIIa. However, the effect of the SSIIa defect on amylose content differs between rice and the other three cereals: as a side effect, the lack of SSIIa function increases amylose content by more than 50% compared to the wild type in wheat, maize and barley, whereas as increase of just 10% occurs in rice. Based on the main effect of amylopectin chain-length, SSIIa-deficient rice grains maintain a softer texture longer than their wild type counterparts after they are cooked or processed into rice cakes. On the other hand, SSIIa-deficient barley grains have a higher content of resistant starch than that in wild-type grains probably because of the large increase in the proportion of amylose in starch, and they have health benefits similar to those of *ae* mutants.

Here, we discovered two rice cultivars that naturally lack starch branching enzyme 1 (BEI) activity in the endosperm. Although induced mutants of starch branching enzyme 1 gene (*Sbe1*) have been screened and characterized, evaluation of the mutants toward a practical use has not been performed. We characterized the starch properties of the naturally-occurring BEI-deficient rice cultivars, and showed that the gene responsible was located within or close to the *Sbe1* locus on chromosome 6. We also developed PCR markers to select for this deficient *Sbe1* allele. BEI-deficient cultivars can be used to improve waxy rice processing.

**MATERIALS AND METHODS**

**Plant materials.** Three rice (*Oryza sativa* L.) cultivars with low pasting temperatures (PTs), Kurnai, Hiderishirazu-D and Tokyo-sensho, were obtained from a collection of upland rice genetic resources. A non-waxy lowland rice, Koshihikari, the leading cultivar in Japan, was used as the control unless otherwise stated. These cultivars were grown in a paddy field at the Ibaraki Agricultural Center or the National Institute of Crop Science, both located in the Ibaraki Prefecture of Japan. For the native-PAGE analysis of BE activity, panicles were harvested at the late milky stage, frozen in liquid nitrogen, and then stored at −30°C until use. For all other experiments, plants were harvested at maturity.

**Measurement and evaluation of starch properties.**

Urea-induced gelatinization of halved rice grains was evaluated as described in the legend of Fig. 1. The distribution of amylopectin chain-lengths was analyzed by fluorescence-assisted carbohydrate electrophoresis by using capillary electrophoresis, as described by Fujita et al. Pasting properties were measured by using an RVA (RVA-4; Newport Scientific Pty Ltd., Sydney, Australia), as described by Umemoto et al. Apparent amylose contents of rice flour were determined by an improved colorimetric method, which will be submitted to the International Organization for Standardization (ISO) as an ISO method.

**Assay of starch branching enzyme activity.** Endosperms in late milky stage were prepared by removing the embryo and pericarp from five or six grains, and then suspended in an extraction medium containing 50 mM HEPES-NaOH (pH 7.4), 4 mM MgCl₂, 50 mM mercaptoethanol and 12.5% (v/v) glycerol on ice and homogenised by a homogeniser. The sample-to-medium ratio was 150 g L⁻¹. The homogenate was centrifuged at 15,000 × G for 5 min at 4°C, and the supernatant was used as the crude enzyme extract. Native-PAGE was performed as described by Nishi et al.

**DNA Sequencing of the *Sbe1* gene and development of PCR markers for *Sbe1* genotyping.** Total DNA was isolated from the leaves of Kurnai, Hiderishirazu-D, Koshihikari and the F₃ progeny derived from the cross of Hiderishirazu-D and Koshihikari, as described by Umemoto et al. DNA sequencing was carried out by using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). The primers used for DNA sequencing, and the PCR markers described below, were designed by using the Primer3 program. We developed three PCR markers based on the sequence polymorphisms between Kurnai/Hiderishirazu-D and Nipponbare/Koshihikari. The primer sequences for detecting the 7-base pair (bp) insertion/deletion (In/De) in the 5'-UTR were *Sbe1* 71/D_F 5'-AAGGAAGGGAGAGAACGGTGAAAGGAAACGTTGAAG-3' and *Sbe1* 71/D_R 5'-GTGCAGCAGTGAGGTGAAAGGAAACGTTGAAG-3'. Those for the 12-bp In/De in the 5'-UTR were *Sbe1* 112/D/F 5'-TTTCTACCTCCACTGGCCTGAC-3' and *Sbe1* 121/D/R 5'-GAGGGTTGAGACAGACAGCATTG-3'. Those for the single nucleotide polymorphism (SNP) in exon 9 were *Sbe1* E9-SNP(G)F 5'-GGATTTAGCCTTCCAAAGAGATGG-3' for the 'G' genotype; *Sbe1* E9-SNP(A)F 5'-GGATTTAGCCTTCCAAAGAGATGG-3' for the 'A' genotype; and *Sbe1* Ex-SNP_R 5'-CAGAAAGAGAAGGAGGATG-3' as the common reverse primer.

**Waxy line breeding and evaluation of rice-cake hardness.** For the production of waxy rice with a high amylopectin short-chain ratio, we used the F₃ population derived from a cross of non-waxy Kurnai and waxy Naebahatamochi. Waxy phenotype grains were selected from husked F₃ seeds, and the selected 159 individuals were cultivated and harvested at

![Fig. 1. Urea gelatinization of halved rice grains.](Image)
maturity. The bulked F₃ grain from each F₂ plant was polished and milled, and the flour was used for RVA measurements.

We selected the three waxy F₃ individuals with the highest PTs, and the three with the lowest PTs, as determined by RVA. These six F₃ individuals were forwarded to the F₄ generation without selection. Six F₃ waxy lines, three upland waxy cultivars (Naebahatamochi, Toyohatamochi and Yumenohatamochi), and two lowland waxy cultivars (Hokurikumochi 210 and Mangetsumochi), were cultivated in a sandy field. Panicles for the native-PAGE analysis of starch branching enzyme activity were harvested as described above. Samples for other experiments were harvested at maturity, and bulked seeds from 10 plants were used.

We made rice cakes by using a mochi-making machine (AFC-10F; Toshiba Corporation, Tokyo, Japan). Processed rice cakes were then placed in an aluminum case (27.5 × 18 × 1.5 cm) and shaped, their surface was covered with polyethylene film, and they were stored at 4°C for 24 h. The hardness of the rice cakes was measured by using a KM-5 fruit hardness meter (Fujiwara Scientific Co., Ltd., Tokyo, Japan).

Rice cake hardness of the BEI-deficient waxy line 'wx/wx/ sbe1' and the waxy line EM583 developed by Satoh et al., which both have a Taichung 65 genetic background, was also evaluated. Eight grams of polished rice was soaked in water overnight. After the water was drained off, the rice was steamed for 30 min. Rice cakes were processed from the steamed rice by using a small-scale mochi-making machine (Minusagi; Food and Nutrition Laboratory, Mishima, Shizuoka, Japan). The machine was run for 8 min, and then the processed rice cake was shaped to a 7 mm thickness by pressing the rice cake in a laboratory dish with 7 mm vertical clearance. Rice cake hardness was measured by using a Texture Analyser (TA-TX2i; Stable Micro System Ltd., Godalming, UK), with five replicates. A cylinder shaped probe (Ø 2 mm) was penetrated into the rice cake to a 3 mm depth, and the maximum load was regarded as the hardness.

**RESULTS AND DISCUSSION**

**Starch properties and chain-lengths distribution of amylopectin.**

In our previous study, we serendipitously recognized that three upland rice cultivars had a lower PT of rice flour than that of other cultivars, and M-type amylopectin, which has the proportion of short-chain between that of S- and L-type. These low PT cultivars were Kurnai, Hiderishirazu-D and Tokyo-sensho. The GT of rice starch increases with ambient temperature during the grain filling period. The PTs as the estimate of the GTs of the three cultivars are less than 68°C, which is rather low when the ambient temperature of the grain-filling period (averaged ambient temperature 20 days after heading > 25°C) is taken into account. Here, we confirmed that the PTs of Kurnai, Hiderishirazu-D and Tokyo-sensho were 65.7, 68.2 and 64.2°C, respectively, and lower than that of Koshihikari (69.5°C), which has S-type amylopectin (Table 1).

The apparent amylose content of Kurnai and Hiderishirazu-D were 15.4 and 17.1%, respectively; these values were not largely different from that of Koshihikari, which was 16.5% (Table 1). In contrast, the apparent amylose content of Tokyo-sensho was 25.8%, which is clearly higher than that of other three cultivars. This difference in amylose content can be explained by the allelic difference in the waxy gene. Tokyo-sensho carries the Wx⁺ allele, whereas the two other low PT cultivars and Koshihikari harbor the Wx⁻ allele, according to the results of a DNA marker analysis developed by Yamanaka et al. (data not shown). Rice with the Wx⁺ allele has a lower amylose content than rice with the Wx⁻ allele, because the amount of Wx protein (GBSSI) in the endosperm is less in the former. Among the viscometric parameters, the peak and breakdown viscosities were clearly lower for the low PT cultivars than for Koshihikari (Table 1). Final and setback viscosities were markedly higher for Tokyo-sensho, which carries Wx⁺, than for the other three cultivars; high values for these parameters are common among Wx⁺ cultivars.

Urea-induced gelatinization of the halved rice grain was tested with the concentration of urea at 2.8, 3.0, 3.2 and 3.4 M, respectively. Among the cultivars, differences in the gelatinization of the grain were clearest at 3.2 M (Fig. 1). Kurnai and Hiderishirazu-D were more susceptible to the 3.2 M urea solution than was Tokyo-sensho, which in turn was slightly more susceptible than was Koshihikari.

The distributions of the amylopectin chain-lengths of Kurnai, Hiderishirazu-D and Tokyo-sensho were compared with that of Koshihikari, which has S-type amylopectin (Fig. 2). Each of the three cultivars with a low PT displayed a higher proportion of short-chains (DP 6–10) and a lower proportion of middle-length chains (DP of 12–20) compared to that of Koshihikari. This result is in accordance with the negative relationship between PT and the amylopectin chain ratio (Σ DP ≤ 10 / Σ DP ≤ 24) reported by Nakamura et al. Since the chain-lengths distribution of amylopectin were similar among Kurnai, Hiderishirazu-D and Tokyo-sensho, the differences in the urea-induced gelatinization might be caused by the differences in amylose content where

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Apparent* amylose content (%)</th>
<th>Pasting* temperature (°C)</th>
<th>Peak viscosity (cP)</th>
<th>Minimum viscosity (cP)</th>
<th>Breakdown viscosity (cP)</th>
<th>Final viscosity (cP)</th>
<th>Setback viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koshihikari</td>
<td>16.5</td>
<td>69.5</td>
<td>4594</td>
<td>1671</td>
<td>2923</td>
<td>3015</td>
<td>1345</td>
</tr>
<tr>
<td>Kurnai</td>
<td>15.4</td>
<td>65.7</td>
<td>3506</td>
<td>1712</td>
<td>1795</td>
<td>3135</td>
<td>1423</td>
</tr>
<tr>
<td>Hiderishirazu-D</td>
<td>17.1</td>
<td>68.2</td>
<td>3079</td>
<td>1548</td>
<td>1532</td>
<td>3097</td>
<td>1550</td>
</tr>
<tr>
<td>Tokyo-sensho</td>
<td>25.8</td>
<td>64.2</td>
<td>3313</td>
<td>1852</td>
<td>1461</td>
<td>3975</td>
<td>2123</td>
</tr>
</tbody>
</table>

*Apparent amylose content was determined by the colorimetric method and the average of triplicated measurements were shown. **Pasting temperature was defined the temperature at which viscosity increased 20 cP in 4 s at the onset of viscosity increase. Viscosity properties, expressed in cP, are the average of two measurements.
condition we used, however lacked activity in vitro. We assume that Tokyo-sensho also has defect in BEI, which presented as the average of three individuals.

Tokyo-sensho had higher amylose content than other two cultivars.

Starch branching enzyme activities.

The differences in chain-length profiles between the low PT cultivars and Koshihikari was very similar to those found between induced mutants lacking BEI activity in the endosperm and their wild type counterparts. Therefore, we conducted Native-PAGE activity staining for BEs. The results clearly showed that, among the three low PT cultivars, Kurnai and Hiderishirazu-D lacked BEI activity, whereas Tokyo-sensho had a similar level of activity to that of Koshihikari (Fig. 3). There were no noticeable differences between cultivars in the activity of BEIIa. The band representing BEIIb activity could not be observed clearly because the band representing starch phosphorylase 1 (Pho1) activity overlapped with it (Fig. 3). We therefore measured the amount of BEIIb protein detected by immunoblotting with antisera raised against purified rice BEIIb. The results confirmed that the BEIIb level was similar between the cultivars lacking and having BEI activity (data not shown).

To our knowledge, Kurnai and Hiderishirazu-D are the first spontaneous BEI-deficient mutants to be identified, not only in rice but in all major cereal crops. We were not surprised that the BEI activity of Tokyo-sensho differed from that of Kurnai and Hiderishirazu-D, because Tokyo-sensho carries the Wx^a allele and the other two cultivars have the Wx^b allele. The Wx^b allele is the major allele among indica subspecies of rice, and the Wx^a allele is predominant in japonica subspecies. (25,27) Tokyo-sensho might be derived from an indica subspecies, and a different evolutionary event may have occurred to cause the short amylopectin chain-lengths. Despite the extensive studies in the function of starch synthesising enzymes including isoamylase 1, BEIIb, BEIIa, starch synthase (SS) I, SSIIIa and Pho1 on the chain-lengths distribution of amylopectin, mutants or knockout lines having the chain distribution similar to those of BEI deficient cultivars have not discovered. (28-31) Therefore, we assume that Tokyo-sensho also has defect in BEI, which has activity in vitro condition we used, however lacked activity in vivo. For example, the BEI of Tokyo-sensho may have an amino acid substitution in the region critical for amylolitic activity. In vitro condition of Native-PAGE activity staining, various lengths of α-polyglucans were synthesized and supplied by the phosphorylase added in the incubation buffer, and transferase activity of BEI in the endosperm of Tokyo-sensho can make α-1,6 bond to synthesize branched polyglucan stained similar to that synthesized by BEI of Koshihikari (Fig. 3). Without the amylolitic activity of its own, α-polyglucans with suitable lengths for the deficient BEI of Tokyo-sensho might not available to synthesize amylopectin side-chains in vivo. Further investigations to identify the cause of the alterations in chain-lengths distribution of amylopectin in Tokyo-sensho will be continued.

Genetic analysis of the distribution of amylopectin chain-lengths.

We measured the amylopectin short-chain ratio, expressed as a percentage (i.e., (Σ DP 6–DP 9/Σ DP 6–DP 24) × 100), in Kurnai and Hiderishirazu-D. Forty-six individual F_2 grains obtained from an F_1 plant that was derived from a cross of Koshihikari and Hiderishirazu-D were analysed together with the parents. The histogram of the short-chain ratios showed a bimodal distribution around a ratio of 15.75%, with a peak of higher ratios corresponding to 11 F_2 seeds and a peak of lower ratios corresponding to 35 F_2 seeds (Fig. 4, upper panel). We then conducted a genetic analysis of the phenotype of high (> 15.75%) amylopectin short-chain ratio. The segregation ratio was fitted to a 1:3 segregation ratio assessed by the chi-square test (χ^2=0.610, df=1, 0.25 < p < 0.50). The result suggested the existence of one recessive gene on the Hiderishirazu-D genome that is associated with the high amylopectin short-chain ratio.

Since Hiderishirazu-D lacked BEI activity in its endosperm, we considered that the Sbe1 gene could be responsible for the BEI deficiency and change in distribution.
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is responsible for the BEI defects and high amylopectin (data not shown). This finding suggests that the same locus than those of Koshihikari and similar to those of the parents, and is polymorphic between Koshihikari and Hiderishirazu-D. When the genotype was Koshihikari homozygote, the short-chain ratio ranged from 14.1 to 14.7%. In contrast, when the genotype was Hiderishirazu-D homozygote, the short-chain ratio ranged from 14.2 to 15.6%, which is a wider range than that of Kurnai homozygotes. Heterozygotes showed ratios ranging from 14.2 to 15.6%, which is a wider range than that found in either homozygote and overlaps with that of Koshihikari homozygotes. Thus, the results showed perfect linkage between the marker genotype and the short-chain ratio. These results strongly support the view that the differences between the short-chain ratios observed in Koshihikari and Hiderishirazu-D were controlled by the Sbe1 locus and amylopectin short-chain ratio with-

of amylopectin chain-lengths. To test this possibility, linkage between the genotype at the Sbe1 locus and amylopectin short-chain ratio was investigated within the F2 population described above (Fig. 4, lower panel). We used a DNA marker based on a SNP that exists in the structural gene of Sbe1 and is polymorphic between Koshihikari and Hiderishirazu-D. When the genotype was Koshihikari homozygote, the short-chain ratio ranged from 14.1 to 14.7%. In contrast, when the genotype was Hiderishirazu-D homozygote the ratio ranged from 15.9 to 17.0%, this range was higher than and did not overlap with the range in Koshihikari homozygotes. Heterozygotes showed ratios ranging from 14.2 to 15.6%, which is a wider range than that found in either homozygote and overlaps with that of Koshihikari homozygotes. Thus, the results showed perfect linkage between the marker genotype and the short-chain ratio. These results strongly support the view that the differences between the short-chain ratios observed in Koshihikari and Hiderishirazu-D were controlled by the Sbe1 locus, or a locus very close to Sbe1.

We further investigated whether the amylopectin short-chain ratios of Hiderishirazu-D and Kurnai, which are higher than that of Koshihikari, are controlled by the same locus. Twenty-four F2 grains derived from a cross of Hiderishirazu-D and Kurnai were analyzed. The results showed that the short-chain ratios of all the F2 grains were clearly higher than those of Koshihikari and similar to those of the parents (data not shown). This finding suggests that the same locus is responsible for the BEI defects and high amylopectin short-chain ratios in Hiderishirazu-D and Kurnai.

The results of genetic analyses support the view that the naturally-occurring polymorphism(s) in Sbe1 of Kurnai and Hiderishirazu-D led to the lack of BEI activity and resulted in the high amylopectin short-chain ratio. The ability of rice BEI to synthesize longer chains of amylopectin than those produced by BEIIb and BEIIa has been clearly demonstrated in vitro experiments. In vivo, BEIIb and BEIIa might compensate for the lack of BEI and make shorter amylopectin chains.

Sequence polymorphisms in the Sbe1 gene.

To examine whether sequence variation(s) that would result in a lack of BEI activity exist in the Sbe1 gene of the Kurnai and Hiderishirazu-D, we sequenced genomic DNA of Sbe1 of these cultivars. The resultant sequences covered the coding region, 5′-untranslated region (UTR), first part of the two 3′-UTRs, and some part of introns. The regions sequenced and the polymorphisms found are shown in Fig. 5, which is based on the genomic DNA sequence of Sbe1 of Nipponbare (Os06g0726400). The Sbe1 sequence of Koshihikari in a database is identical to that of Nipponbare except that a part of sequence in intron 2 has not confirmed. The grey bar corresponds to the region containing the Sbe1 gene. Rectangles in white and black are untranslated regions and exons, respectively. Black lines show the region where the genomic DNA sequences of Kurnai and Hiderishirazu-D were determined in this study. Approximate positions and type of sequence variations are shown, except for those in introns. In/Del, insertion/deletion.

short-chain ratios in Hiderishirazu-D and Kurnai.

The grey bar corresponds to the region containing the Sbe1 gene. Rectangles in white and black are untranslated regions and exons, respectively. Black lines show the region where the genomic DNA sequences of Kurnai and Hiderishirazu-D were determined in this study. Approximate positions and type of sequence variations are shown, except for those in introns. In/Del, insertion/deletion.
In/Del polymorphisms in the 5'-UTR and the SNP causing an amino acid substitution in exon 9 (Fig. 6).

We identified no obvious loss-of-function variations such as a frame shift mutation that would introduce an earlier stop codon. Among the nine sequence variations detected were two In/Del polymorphisms in the core promoter region of the gene; however, neither of these affects the sequences of transcription factor binding sites, such as the TATA box or CAAT box. Of the two exonic SNPs, the one in exon 9 results in the amino acid substitution of 607Gly to 607Asp. However, this substitution is not critical for BEI activity, because additional sequencing of this region in Naebahata showed differences in GT and several viscosity parameters. 26) 37)  examined a different set of alleles and gelatinization and viscosity parameters. 26,36)  The possible functional variations identified in the above association studies need to be confirmed, for example, by breeding a near isogenic line for the target allele. 54,51)  The fact that the phenotype of the BEI-deficient grain cannot be distinguished from a cross of Naebahatamochi and Kurnai. However, both Kurnai and Hiderishirazu-D are non-waxy rice. To investigate if the BEI deficiency that results in a low PT, amylose content, nitrogen content and retrogradation of gelatinized starch, however, other studies did not detect significant associations between Sbe1 alleles and gelatinization properties. 36,38)  The possible functional variations identified in the above association studies need to be confirmed, for example, by comparing the enzyme activity between alleles.

Utilization of Sbe1 deficiency in waxy rice.

The effects of altered amylopectin chain-lengths on processing might be magnified in waxy rice because amylopectin is the sole component of the grain starch. However, both Kurnai and Hiderishirazu-D are non-waxy rice. To investigate if the BEI deficiency that results in a higher amylopectin short-chain ratio affects the processing quality of waxy rice, we crossed Kurnai with a waxy cultivar, Naebahatamochi. The waxy homozygous F2 progeny derived from a cross of Kurnai and Naebahatamochi were screened by observing the phenotype of the F3 grains from each plant. Among the waxy F2 progenies, the three individuals with the highest and three with the lowest PT of rice flour were selected, and the descendant F3 lines were cultivated. By using the bulked F3 grains harvested from the F3 lines, we analysed BEI activity in the developing endosperm. BEI activity was detected in the three lines with high PT, but not in the three lines with low PT (Fig. 7).

Rice cakes were made from the grain harvested from
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After the rice cakes were stored at 4°C for 24 h, their hardness was measured by using a fruit-hardness meter (see Experimental section). The amylopectin short-chain ratio was calculated as $\Sigma$ DP 6-DP 9/ $\Sigma$ DP 6-DP 24, and expressed as a percentage. Empty diamonds, F$_5$ lines without BEI activity; grey diamonds, F$_5$ lines with BEI activity; and black diamonds, Naebahatamochi (waxy donor of the F$_5$ lines). Crosses, waxy rice cultivars (Toyohatamochi, Yumenohatamochi, Mangetsu-mochi and Hokurikumochi 210).

These F$_5$ lines and five waxy cultivars including Naebahatamochi. The relationship between the amylopectin short-chain ratio and rice-cake hardness measured 24 h after processing was investigated (Fig. 8). The three F$_5$ lines lacking BEI activity clearly had higher amylopectin short-chain ratios and a softer texture of rice cake compared to the three F$_5$ lines with BEI activity and the other waxy cultivars. To confirm that BEI deficiency was the cause of the softness of the rice cakes, we further evaluated the process of hardening of rice cakes with a wx mutant line (EM583) and a wx/sbe1 double mutant line with the same genetic background (Taichung 65)$^{(60)}$ (Fig. 9). The hardness of rice cakes made from the wx/sbe1 line was similar to those made from the wx line just after processing. However, hardness developed quicker and further in rice cakes made from the wx line than those made from the wx/sbe1 line. After 5 h storage at 5°C, significant differences were observed. After 24 h rice cakes made from the wx line were more than three times the hardness of those made from the wx/sbe1 line.

The hardening speed of the rice cake is one of the important factors that determines the utilization of waxy rice. Waxy rice cultivars that produce rice cakes that become hard quickly after processing are suitable for the manufacture of simple rice cakes or rice cakes used for rice cracker production, because rice cakes are difficult to cut when they are soft and sticky. In contrast, waxy rice cultivars that produce rice cakes that keep soft for longer periods are suitable for Japanese-style sweets, such as soft rice cake stuffed with sweetened bean jam. Here, we bred and used waxy rice lines with BEI deficiency and also used another source of BEI-deficient materials that have been genetically and biochemically well characterized,$^{(60)}$ and showed that BEI deficiency has a function in maintaining the softness of processed rice cakes.

Confectioners use some sugars, including sucrose and trehalose or starch-degrading enzymes, to retain the softness of rice cakes for Japanese-style confectionary. Rice cakes produced from BEI-deficient cultivars could potentially keep the same softness without the use of sugars and food additives. Retaining the softness of rice cakes has double merit: it benefits consumers because the eating quality is maintained; and it benefits confectioners and retailers because the shelf life of the product is extended. To develop commercial waxy and non-waxy cultivars lacking BEI, a breeding program that uses the BEI-deficient cultivars presented here, and the PCR markers developed here, is currently underway in several agricultural stations in Japan.

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