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Research Paper

Genetic region responsible for the differences of starch properties in two glutinous rice cultivars in Hokkaido, Japan

Tomohito Ikegaya*[1)] and Kanae Ashida[1,2)]

[1)] *National Agriculture and Food Research Organization (NARO), Hokkaido Agricultural Research Center*, Sapporo, Hokkaido 062-8555, Japan

[2)] *Present address: NARO, Institute of Food Research*, Tsukuba, Ibaraki 305-8642, Japan

Starch properties are major determinants of grain quality and food characteristics in rice (*Oryza sativa* L.). Control of starch properties will lead to the development of rice cultivars with desirable characteristics. We performed quantitative trait locus analysis and detected a putative region on chromosome 2 associated with phenotypic variation of starch properties in two glutinous rice varieties developed in the Hokkaido region of Japan: 'Kitayukimochi', which has a low pasting temperature and creates soft rice cakes, and 'Shirokumamochi', which has a high pasting temperature and creates hard rice cakes. *Starch branching enzyme IIb* (*SbeIIb*) was identified as a candidate gene within the region. Sequence analysis of *SbeIIb* in parental lines identified two single-nucleotide polymorphisms (SNPs) with non-synonymous mutations in the coding region of the 'Shirokumamochi' genotype (*SbeIIb*^{sr}). We genotyped over 100 rice cultivars, including 28 rice varieties in the Honshu region of Japan, using the CAPS marker, which was designed using one of the SNPs. However, *SbeIIb*^{sr} was not found in rice cultivars in Honshu. Distribution analysis indicated that *SbeIIb*^{sr} was introduced to the rice breeding population in Hokkaido from the American variety 'Cody' via the Hokkaido cultivar 'Kitaake'. As a result, *SbeIIb*^{sr} was distributed only in progenies of 'Kitaake'.

Key Words: glutinous rice, rice cake hardness, pasting temperature, *Starch branching enzyme IIb*, local breeding population.

Introduction

Starch is the major nutrient component of rice (*Oryza sativa* L.). Most plant starches consist of a mixture of linear and highly branched polymers called amylose and amylopectin, respectively. The molecular structures of amylose and amylopectin are distinctly different among plant species and varieties. In rice, the amylose content and chain length distribution of amylopectin branches determine the physicochemical properties of starch and the cooking properties of grains through changes in gelatinization and starch retrogradation. Glutinous rice has a null mutation with a non-functional granule-bound starch synthase I protein, and it has no amylose (Wang *et al.* 1995). Therefore, glutinous rice is suitable for analyzing the effect of amylopectin properties on food characteristics such as processing, cooking, and consumption properties. The molecular structure of amylopectin has a great influence on the starch properties of glutinous rice (Suzuki *et al.* 2006). Igarashi *et al.* (2008) reported that a higher amylopectin short chain ratio results in a lower rice cake hardness. The chain length distribution of amylopectin is determined by the interactions of starch-metabolizing enzymes such as starch synthase and starch branching enzyme.

Mutant lines that have a defect in genes encoding starch-synthesizing enzymes show distinctly altered starch properties from the wild type. Therefore, natural and induced mutants have been utilized for the analysis of starch synthesis genes and their interactions that control the chain length distribution of amylopectin. In the case of the *amylose extender* (*ae*) mutant 'EM10', which lacks *starch branching enzyme IIb* (*SbeIIb*) activity, the amylopectin short chain (degree of polymerization (DP) < 17) proportion is decreased and the middle length chain (DP 18–35) proportion is increased (Nishi *et al.* 2001), which results in a higher gelatinization temperature (Jane *et al.* 1999). Rice mutants that lack *starch branching enzyme 1* (*Sbe1*) activity are rich in short chains of amylopectin and have a low pasting temperature of rice flour (Okamoto *et al.* 2013, Satoh *et al.* 2003a). A lack of *starch synthase IIa* activity causes changes in the chain length distribution of amylopectin; the proportion of short chains (DP 7–9) is enriched, and the proportion of middle length chains (DP 12–20) is depleted

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*Corresponding author (e-mail: tikegaya@affrc.go.jp)

(Umemoto *et al.* 2002). Based on these studies, 'Aichi-mochi 126', with a genotype lacking *Sbe1* activity, and 'Chikushi-kona 85', which inherited its starch property from 'EM129' (a mutant line deficient in *SbeIIb*), were developed in recent years (Suzuki *et al.* 2019, Wada *et al.* 2018).

Although analyses using mutants and the development of cultivars with novel starch properties have previously been performed, the differences in the molecular structure of amylopectin among elite rice cultivars have not been analyzed. There are fewer differences in the molecular structure of amylopectin among elite rice cultivars than there are in function-deficient mutants. In addition, the chain length distribution of amylopectin is affected by environmental conditions such as temperature at the maturation stage (Igarashi *et al.* 2008). This makes it difficult to select elite rice cultivars and experimental conditions for appropriate analysis. However, an understanding of the genetic mechanisms underlying the differences in starch properties among elite rice cultivars will facilitate the development of more desirable varieties, since elite cultivars have superior agricultural characteristics such as increased yield, tolerance to environmental conditions, and high food quality. In this study, we selected two glutinous rice cultivars developed in Hokkaido, Japan (41–45° N), 'Kitayukimochi' (Shinada *et al.* 2016) and 'Shirokumamochi' (Kasuya *et al.* 2013). Both cultivars show nearly the same phenotypes for the number of days to heading and maturing (Kasuya *et al.* 2013, Shinada *et al.* 2016). 'Kitayukimochi' has a low pasting temperature and hardness, and it is often used in Japanese sweets or refrigerated sweets to ensure softness after cooking and freeze-thawing. On the other hand, 'Shirokumamochi' has a higher pasting temperature and hardness, and it is suitable for making cubed or cylindrical rice cakes. 'Kitayukimochi' and 'Shirokumamochi' exhibit different starch properties even when cultivated in the same environmental conditions, so these varieties are considered to be suitable for detecting the candidate gene for differences in starch properties. This study conducted a distribution analysis targeting a SNP on the candidate gene to reveal the process for its introgression to the local rice population in Hokkaido. This knowledge may help in developing a breeding strategy for expanding the genetic and phenotypic diversity of the local rice population.

Materials and Methods

Plant material and growth conditions

'Kitayukimochi' and 'Shirokumamochi' were cultivated and harvested in 2012 and 2013. Seeds were used for analyses of starch properties (2012) and chain-length distributions (2012, 2013). We crossed 'Kitayukimochi' and 'Shirokumamochi' to obtain F_2 individuals. To detect quantitative trait loci (QTLs) for starch properties, we cultivated 96 F_2 plants and developed F_3 populations by self-pollination in 2018. The F_3 populations were cultivated in 2019.

Some of the rice varieties used in distribution analysis were obtained from Genebank, Japan (https://www.gene.affrc.go.jp/index_j.php). Twenty-eight varieties widely cultivated in Honshu, Japan were selected (**Supplemental Table 1**). In addition, the Hokkaido Rice Core Panel (Shinada *et al.* 2014) and Hokkaido elite rice cultivars were used for the distribution analysis (**Supplemental Table 2**).

All materials were planted in experimental paddy fields at the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Sapporo, Japan (43°00′ N, 141°42′ E) under natural conditions. Sowing and transplanting were performed in late April and late May, respectively. Cultivation management followed the standard procedures used at the Hokkaido Agricultural Research Center. Young leaves were sampled for DNA extraction. Seeds were harvested during the full maturity stage.

Analysis of starch properties

Starch purification: 2 g of polished rice were soaked in 20 mL of 0.1% NaOH overnight at 4°C and ground using a mortar and pestle. The ground rice was filtered through a 70 μm cell strainer (Falcon) and then washed with chilled Milli-Q water by repeating suspension and centrifugation until neutralized. The washed starch was then freeze dried and defatted by heating in methanol.

Analysis of chain-length distribution: The chain length distribution of amylopectin was determined by using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) based on Yasui *et al.* (2009). The purified starch (5 mg) was gelatinized with 5.0 mL of Milli-Q water at 100°C for 1 h with intermittent swirling and was then cooled in water at room temperature (20–25°C). Fifty microliters of 0.5 M sodium acetate buffer at pH 4.7, 10 μL of 2% sodium azide solution, and 5 μL of isoamylase (Hayashibara, Japan) were added to 930 μL of gelatinized starch solution and incubated with stirring at 40°C for 24 h. After incubation, 60 mL of 30% ammonia solution with Milli-Q water (1:4) was added to alkalize the solution, 300 mL of 1% sodium borohydride in 30% ammonia solution with Milli-Q water (1:29) was added to reduce isoamylolyzates, and the tube was allowed to stand overnight. Two 600 mL aliquots of the solution were transferred into two 2 mL microcentrifuge tubes and freeze dried. One hundred and fifty microliters of 1 M NaOH were added, and the mixture was vortexed and allowed to stand for 1 h to dissolve completely. It was then diluted with 850 μL Milli-Q water. The solution was filtered using Ultrafree-MC (0.20 μm, Millipore). The filtrate (25 μL) was separated by HPAEC-PAD (ICS-3000, Dionex, Sunnyvale, CA, USA) with a guard column (4 mm i.d., 650 mm) and a CarboPak PA1 analytical column (4 mm i.d., 6250 mm; Dionex, Sunnyvale, CA, USA) at 35°C with a gradient composed of 0.15 M aqueous NaOH (eluent A), and 0.15 M aqueous NaOH plus 0.5 M sodium acetate (eluent B), at a flow rate of 1.00 mL/min. The separation gradient was:

–10–0 min (before injection), 90% A and 10% B; 0–60 min, linear gradient to 100% B; 60–70 min, 100% B. Polished rice was ground using a sample mill with a 0.5 mm screen (ZX200, Retsch GmbH, Haan, Germany). The moisture content of milled rice flour was calculated as weight loss after drying at 135°C for 1 h.

Pasting properties: Pasting temperature was measured using a Rapid Visco Analyzer (RVA3D+, Newport Scientific Pty Ltd., NSW, Australia). For a rice flour suspension, 4.0 g of rice flour and 20.5 mL Milli-Q water (14% moisture basis) were used. The phenotypic values were measured without inactivation of alpha-amylase in order to measure the difference in each varieties (strains) under natural conditions for making the rice cake. The test profile STD2 was modified and used by heating the rice flour suspension to 95°C and maintaining it at that temperature for 2 min. A constant rotating paddle (160 rpm) was used.

Hardness of rice cake: After measuring the pasting temperature using an RVA, rice flour pastes were placed in a stainless petri dish (φ5.0 × height 1.5 cm) with a cover and stored at 4°C for 24 h. The chilled rice flour paste was considered a rice cake substitute. The hardness of the rice flour paste was measured using the creep meter RHEONER II RE2-33005C (Yamaden, Japan) equipped with a 20 N load cell. The rice flour pastes were uniaxially compressed with a cylindrical resin probe (16 mm diameter) to 30% of the original thickness at a constant rate of deformation (1.0 mm/s). Firmness was calculated as the compressive stress required for 30% strain.

Re-sequencing

Genomic DNA of ‘Shirokumamochi’, extracted from 30 mg leaves, was used for pair-end sequencing by Illumina Hiseq 4000. Raw sequence data of ‘Shirokumamochi’ were deposited in the DDBJ BioProject database under the accession number PRJDB10840. To ensure their quality, raw data were modified by the following two steps: adapter sequences were deleted, and the reads containing low-quality bases (quality value ≤30) were removed. The trimmed reads were aligned onto the reference genome Os-NIPPONBARE-Reference-IRGSP-1.0 (Kawahara *et al.* 2013) using BWA Burrows-Wheeler Aligner (Li and Durbin 2009a), SAMtools (Li *et al.* 2009b), Genome Analysis Toolkit (McKenna *et al.* 2010), and Picard (http://broadinstitute.github.io/picard/). After alignment on the reference genome, structural variations and indels were predicted using BrakeDancer and Pindel. Polymorphisms containing a sequence depth <5 and a heterozygous genotype were considered to be of low quality and were subsequently removed. SnpEff with the default setting was used to annotate these polymorphisms based on their genomic locations, such as whether each was an intron, untranslated region (5′ or 3′), upstream (1000 bp), downstream (500 bp), coding sequence, splice site, or intergenic region on the NIPPONBARE reference genome in Michigan State University’s (MSU) Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/). The sequence data of ‘Kitayukimochi’ was provided by the Hokkaido Research Organization, who holds the breeding rights for this variety. A total of 71,717 SNPs was detected between ‘Kitayukimochi’ and ‘Shirokumamochi’. From these SNPs, total 96 SNP markers that have been used at the Advanced Genomics Breeding Section of the Institute of Crop Science, NARO (NICS) and covered the entire 12 chromosomes, were tested and finally 82 SNP markers were selected.

DNA isolation and genotyping

Total DNA was isolated from young leaves using the CTAB method (Murray and Thompson 1980). Single nucleotide polymorphism (SNP) markers showing polymorphism between the parental lines were selected by comparing whole genome sequences, and the genotypes were detected using the Fluidigm EP1 System (https://dnatech.genomecenter.ucdavis.edu/fluidigm-ep1/).

Detection of QTLs

The program MAPMAKER/EXP 3.0 (Lander *et al.* 1987), based on the Kosambi function (Kosambi 1944), was used to build a linkage map. QTL analysis was performed using composite interval mapping provided by QTL Cartographer v. 2.5 software (Jansen and Stam 1994, Wang *et al.* 2012, Zeng 1994). Genome-wide threshold values ($\alpha = 0.05$) were calculated from the results of 1000 permutations for QTL detection.

DNA sequencing analysis of the SbeIIb gene and development of CAPS marker for SbeIIb genotype

Polymorphic SNPs 2 kbp upstream and downstream of *SbeIIb* were determined by comparing the whole genome sequences of parental lines. One of the SNPs was converted to a cleaved amplified polymorphic sequences (CAPS) marker using the Primer3 program (https://primer3.ut.ee/). The CAPS marker obtained thereby was SbeIIb Ex3-1 (F: 5′-TTGCTTGTTGTCGCTCATTC-3′ and R: 5′-CTCCT GTTGGTGGGACAACT-3′). The PCR reaction mixture consisted of 25 ng of total DNA, each dNTP at 200 mM, 20 pmol of primer, and 2 units of Taq DNA polymerase with PCR buffer (Promega, USA) in a 15.0 μL volume. The thermocycler was programmed of 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final step of 72°C for 7 min. Amplified products of this CAPS marker were digested with restriction enzyme BspT107I (HgiC I) and electrophoresed on a 2.0% agarose gel.

Results

Chain length distribution patterns and starch properties

To clarify the amylopectin structure in the two glutinous rice cultivars from Hokkaido, the chain-length distribution was analyzed. The side chains were classified into four

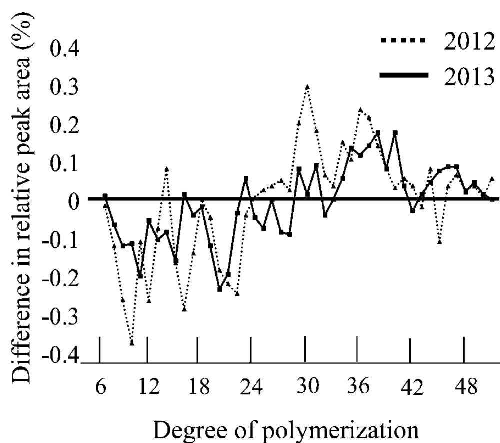

Fig. 1. Comparison of chain-length distribution profiles of amylopectin from 'Kitayukimochi' and 'Shirokumamochi'. The difference in the profiles was calculated by subtracting the ratio of a chain of given length of 'Kitayukimochi' from that of the value of 'Shirokumamochi'. Values are the means of two replicates in 2012 and 2013, respectively.

groups according to Hanashiro *et al.* (1996). In the amylopectin chain-length distribution patterns of 'Shirokumamochi' compared to 'Kitayukimochi' the proportions of side chains with DP 6–12 and DP 13–24 were significantly decreased in 2012 and 2013, and the proportion of side chains with DP 25–36 was significantly increased in 2012 (**Fig. 1**, **Table 1**). The pasting temperature and hardness of rice paste were significantly higher in 'Shirokumamochi' than in 'Kitayukimochi' in 2012, 2018 and 2019, respectively (**Table 2**).

QTLs for pasting temperature and hardness of rice flour paste

The phenotypic distributions of pasting temperature and hardness of rice cake in the F_2 population were shown (**Supplemental Fig. 1**). Eighty-two SNP markers were used to genotype 12 chromosomes of the F_2 populations, and QTL analysis was performed to assess pasting temperature and hardness of rice flour paste (**Table 3**, **Supplemental Table 3**). QTLs were detected in the same region of chromosome 2. The QTLs were mapped in the interval between the SNP markers FA0159 and FA0817 in the region around 7.4 Mbp, and the nearest marker was FA0802. The logarithm of the odds (LOD) scores of the QTLs were 6.33 and 4.25 for pasting temperature and rice paste hardness, respectively. The percentages of phenotypic variation explained were 41.2% and 17.0% for pasting temperature and hardness of rice flour paste, respectively. The additive effects of 'Kitayukimochi' allele were –0.48 (°C) and –0.11 (N) for pasting temperature and hardness of rice flour paste, respectively. The 'Kitayukimochi' alleles lowered the pasting temperature and kept rice flour paste soft.

Table 1. HPAEC-PAD fractions of debranched amylopectin from 'Kitayukimochi' and 'Shirokumamochi'

Variety	Cultivated year	DP 6–12 (%)	DP 13–24 (%)	DP 25–36 (%)	DP 37–60 (%)
Kitayukimochi	2012	26.2 **	47.1 **	11.8 ***	14.8
Shirokumamochi		25.0	46.0	13.5	15.6
Kitayukimochi	2013	25.8 **	47.2 **	12.4	14.7
Shirokumamochi		25.1	46.3	12.9	15.7

* indicates significant difference (Student's t-test, ** P < 0.01, *** P < 0.001).

Table 2. Starch properties in 'Kitayukimochi' and 'Shirokumamochi'

Variety	Cultivated year	Pasting temperature (°C)	Hardness (N)
Kitayukimochi	2012	62.8 *	2.16 ***
Shirokumamochi		63.6	2.46
Kitayukimochi	2018	61.8 **	2.14 ***
Shirokumamochi		63.5	2.47
Kitayukimochi	2019	66.7 **	2.17 ***
Shirokumamochi		67.8	2.52

* indicates significant difference (Student's t-test, * P < 0.05, ** P < 0.01, *** P < 0.001).

Table 3. Putative QTL for pasting temperature and hardness

Nearest marker	Chromosome	Position (Mbp)	LOD	Var.exp (%)	Additive effect[a]	Phenotype
FA0802	2	15.3–22.8	6.33	41.2	–0.48 (°C)	pasting temperature
			4.25	17.0	–0.11 (N)	hardness

[a] Additive effect of 'Kitayukimochi' allele.

Breeding Science
Vol. 71 No. 3 BS

Fine mapping and screening of candidate genes for starch properties

The recombinant F_3 lines in the candidate region were used to delimit the locus for starch properties. Six lines, recombinant between the markers FA2431 and FA2438, were genotyped using an additional 52 SNP markers (data not shown), and starch properties were compared (**Fig. 2a**). Each pairing of F_3 lines No. 25 & 24, 12 & 13, and 66 & 65 originated from the same F_2 recombinant individuals. The genotype of two markers, FA5957 and FA5969, in a 305 kbp region was associated with pasting temperature and hardness of rice flour paste (**Fig. 2a**). For screening candidate genes, MSU Rice Genome Annotation Project database was used. MSU predicts 34 protein-coding genes in the 'Nipponbare' reference genome of this 305 kb region. 'Nipponbare' is a standard cultivar of non-glutinous rice in Japan. Among these 34 candidate genes, one gene encodes *Starch branching enzyme IIb* (*SbeIIb*; Os02g32660.1). In the 'Nipponbare' reference genome, *SbeIIb* encompasses 8656 bp, comprising 22 exons consisting of 1914 bp and encoding a predicted protein with 638 amino acid residues. Genome sequence analysis showed that the sequence of *SbeIIb* in 'Kitayukimochi' is the same as in 'Nipponbare' (**Supplemental Table 4**). Comparing the *SbeIIb* DNA sequence between 'Kitayukimochi' and 'Shirokumamochi' revealed that G-to-T (Leu94Val) and G-to-A (His196Arg) mutations existed at the third and fourth exons in 'Shirokumamochi'. These mutations were predicted to result in non-synonymous substitutions of amino acids (**Fig. 2b**). The G-to-T mutation generated a recognition site for the restriction enzyme BspT107I (HgiC I). We developed a CAPS marker SbeIIb Ex3-1 (**Fig. 3**) to determine the genotypes of the plants. The genotype of the 'Shirokumamochi' determined by SbeIIb Ex3-1 was named *SbeIIb*^{sr}. Mutations were found between 'Kitayukimochi' and 'Shirokumamochi' in exons, introns, and each 2 kbp region upstream and downstream of *SbeIIb* (**Supplemental Table 4**).

Distribution analysis targeting a SNP on the candidate gene

To examine the distribution of a SNP on the candidate gene in the genetic region responsible for pasting temperature and hardness of rice cake, the CAPS marker SbeIIb

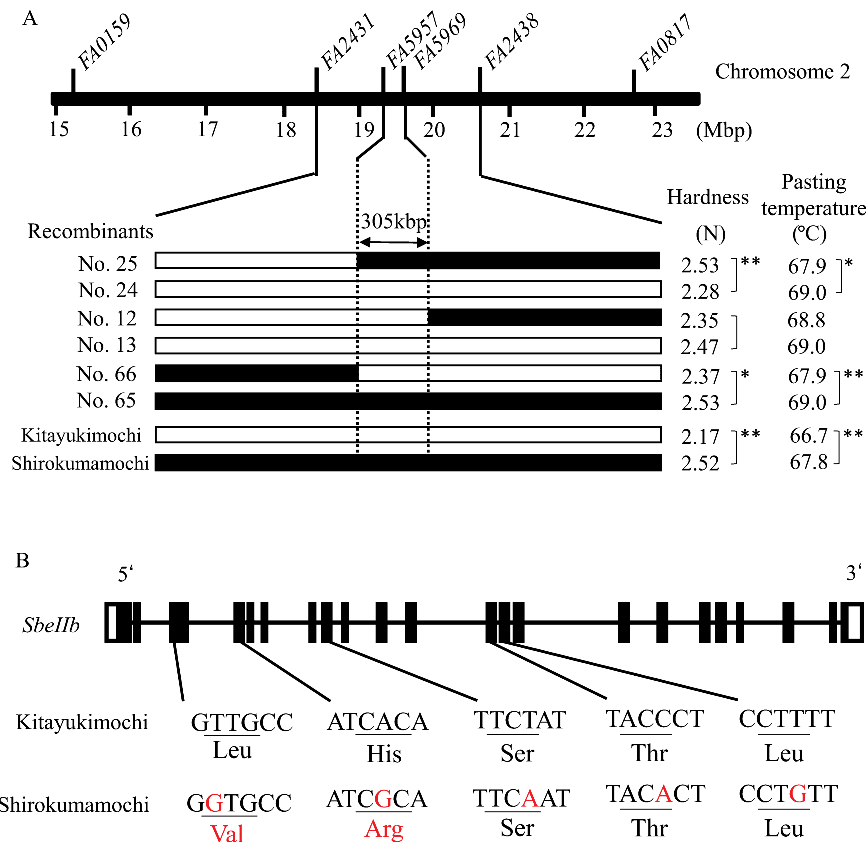

Fig. 2. Genetic map around the QTL. A. Additional genetic mapping of F_3 recombinants and progeny testing. Recombinants between marker loci FA2431 and FA2438 were selected from 96 F_2 plants. White bars indicate homozygous 'Kitayukimochi' and black bars indicate homozygous 'Shirokumamochi'. Each pairing of No. 25 & 24, 12 & 13, and 66 & 65 originated from the same F_2 recombinant individual. * and ** mean 1% and 0.1% sufficiency according to t-test, respectively. B. Schematic diagrams of *SbeIIb* gene. Open and black boxes indicate untranslated regions and exons, respectively. Letters indicate DNA sequences of exons and introns, respectively. Translated amino acids are shown under the DNA sequence. Substituted nucleotides and amino acids are shown by red letters.

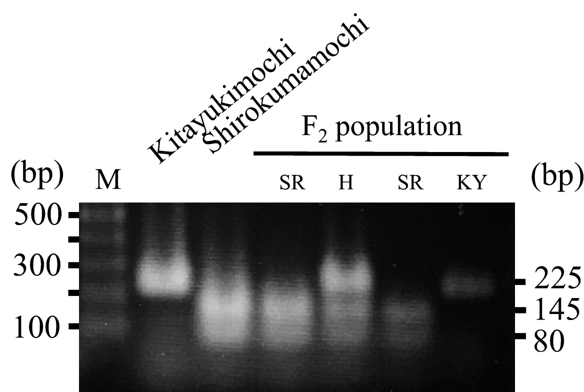

Fig. 3. Band patterns of digested DNA. Amplicon lengths are indicated on the right. M means size marker. KY: 'Kitayukimochi', SR: 'Shirokumamochi', H: Hetero.

Ex3-1 was used. First, we genotyped 28 rice varieties in Honshu, Japan, including 17 glutinous and 11 non-glutinous varieties (**Supplemental Table 1**). However, *SbeIIb*^{sr} was not detected. The *SbeIIb* genotypes of Japanese native rice core collection (JRC) were determined by referring to the TASUKE (https://ricegenome-corecollection.dna.affrc.go.jp/) database. Only indica type variety JRC42 'Touboshi' had 5 SNPs on exon 3, 4, 8 and 12 same as 'Shirokumamochi'. However, other SNPs in introns in 'Touboshi' were not completely matched to 'Shirokumamochi'. According to the rice variety database (https://ineweb.narcc.affrc.go.jp/), the progeny of 'Touboshi' has not exist in rice cultivars in Japan. Analysis of the Hokkaido rice core panel (63 varieties), 16 elite rice cultivars (10 varieties overlapping with the core panel) in Hokkaido, and ancestral varieties of 'Shirokumamochi' indicated that *SbeIIb*^{sr} exists only in the cultivars of 'Kitaake' progenies in Hokkaido (**Fig. 4**, **Supplemental Table 2**). To confirm whether the 'Shirokumamochi' genotype was really derived from 'Cody', we compared the 'Shirokumamochi' sequence with the 'Kitaake' sequence (DRP004087) from DDBJ. The sequences of both varieties matched perfectly. The origin of *SbeIIb*^{sr} was predicted to be 'Cody', an American variety. *SbeIIb*^{sr} was introduced via the cultivar 'Kitaake' to Hokkaido elite lines. *SbeIIb*^{sr} was introduced to almost all non-glutinous elite rice cultivars in Hokkaido (12/13 varieties; **Supplemental Table 2**).

Discussion

We observed significant differences in chain-length distribution patterns, pasting temperature, and hardness of rice flour paste between two glutinous local elite rice cultivars, 'Kitayukimochi' and 'Shirokumamochi', in Hokkaido (**Fig. 1**, **Tables 1**, **2**). The relationship between the pasting temperature and hardness of rice cake was differed depending on the year of cultivation (**Table 2**) and did not show linear correlation. Because not only the amylopectin structure but also the amylase activity affects the hardness of the rice cake, the value of the pasting temperature and the hardness of the rice cake do not always have a linear relationship. We have conducted single-year QTL analysis in F_2 population and detected one QTL (**Table 3**). However, phenotypic values in the F_2 population showed continuous variation (**Supplemental Fig. 1a**, **1b**). The cause could be heading date fluctuation between F_2 individuals (up to 2–3 days) and other minor genetic factors other than on the candidate region. These factors would be able to be detected more accurately by performing a multiple-year QTL analysis. Additional mapping using F_3 recombinants revealed a 305 kbp putative region associated with differences in pasting temperature and hardness of rice flour paste (**Table 3**, **Fig. 2a**). *SbeIIb*, which codes for the starch branching enzyme, was identified as a candidate gene within the region. Sequence analysis of *SbeIIb* in

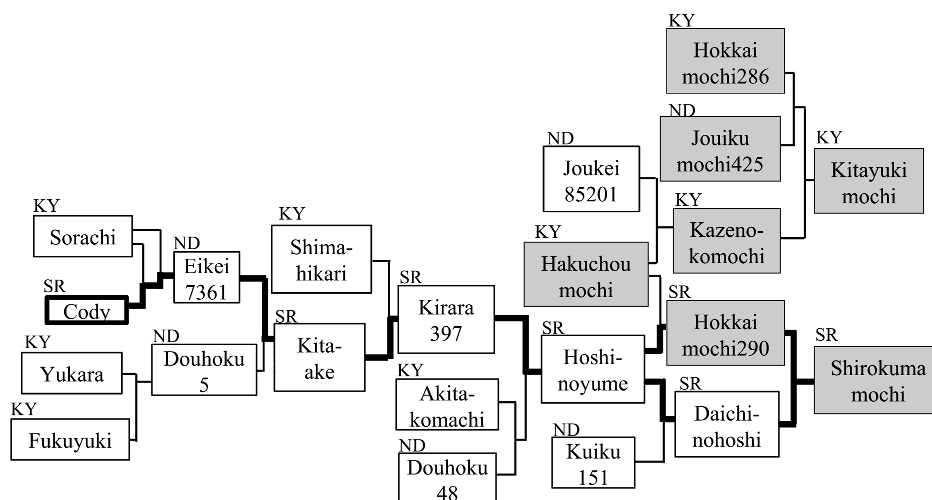

Fig. 4. The pedigree of 'Kitayukimochi' and 'Shirokumamochi'. Gray boxes indicate glutinous rice, and white boxes indicate non-glutinous rice. Letters represent the results genotyped by CAPS marker SbeIIb Ex3-1. KY and SR represent the genotype of 'Kitayukimochi' and 'Shirokumamochi', respectively. ND means 'not determined'. The thick black lines indicate the way *SbeIIb*^{sr} was inherited.

'Kitayukimochi' and 'Shirokumamochi' identified two missense mutation and three silent mutation sites in the coding region (**Fig. 2b**). *SbeIIb* plays a role in the formation of short chains within the amylopectin cluster during starch synthesis (Nishi *et al.* 2001). The non-glutinous rice mutant 'EM10', the *amylose extender* (*ae*) mutant, contains starch with amylose and amylopectin branches longer than those of the wild type as a result of *SbeIIb* deficiency (Mizuno *et al.* 1993, Nishi *et al.* 2001, Satoh *et al.* 2003b, Yano *et al.* 1985). The starch in *ae* has a higher gelatinization temperature, with a decreased proportion of short amylopectin branches, than wild-type starch (Butardo *et al.* 2011, Nishi *et al.* 2001, Satoh *et al.* 2003b, Yano *et al.* 1985). In addition, starch from *ae* and *waxy* double mutants has a higher gelatinization temperature than that of wild or single mutation strains (Kubo *et al.* 2010, Nishi *et al.* 2001). This suggests that the differences in starch properties between the two glutinous rice varieties used in this study could be derived from alteration of the *SbeIIb* gene.

The *ae* mutant 'EM10' contains a substantial amount of resistant starch (Ohtsubo *et al.* 2006) and is expected to be a useful material for a low glycemic-index diet. However, 'EM10' also has a deteriorated grain appearance and a low grain weight. The yield of 'EM10' is 60–70% of its wild-type cultivar equivalent, 'Kinmaze', and thus, it is not suitable for commercial production in the rice market. To enable fine-tuning of starch characteristics without degrading yield or eating quality, the non-glutinous rice mutant lines *altered gelatinization (age)1* and *age2*, with moderate differences in starch gelatinization temperature, were developed (Nakata *et al.* 2018). Lines *age1* and *age2* have a non-synonymous substitution (M723K) at the 20th exon and insertion of a retrotransposon, Tos17, in the 17th intron in *SbeIIb*, respectively. These lines exhibit a higher gelatinization temperature with a decreased proportion of short amylopectin branches than wild-type starch at different levels in each strain. The effects on grain quality and amylopectin structure in *age* alleles are significantly weaker than those in 'EM10' (Nakata *et al.* 2018). However, it is unclear whether *age1* and *age2* are suitable for commercial production because there is currently no information on yields or other agricultural traits.

Developing a new rice variety using mutant lines requires time and labor to confirm whether the target trait is improved as desired and whether other agricultural traits are suitable for the environmental conditions of the cultivation area. 'Shirokumamochi' and 'Kitayukimochi', the varieties used in this study, were developed as elite cultivars in Hokkaido with superior agricultural characteristics such as yield, cold tolerance, grain appearance, and taste (Kasuya *et al.* 2013, Shinada *et al.* 2016). It is presumed that the genotype *SbeIIb*^{sr}, identified in this study, does not have a negative effect on rice production.

The genotype *SbeIIb*^{sr} was first introgressed from the exotic germplasm 'Cody' to the non-glutinous rice cultivar 'Kitaake' and then introduced into 'Shirokumamochi' through the non-glutinous elite rice cultivars 'Kirara397', 'Hoshinoyume' and 'Daichinohoshi', progenies of 'Kitaake' (**Fig. 4**). Because 'Hoshinoyume' is a non-glutinous rice variety, it is presumed that the purpose of this crossing was not to modify the starch properties of glutinous rice, but to improve other agricultural traits such as yield, cold tolerance, and grain appearance. Nevertheless, glutinous rice cultivars with high pasting temperature and rice paste hardness were selected. This may be an unexpected breakthrough about starch properties in the Hokkaido breeding population.

SbeIIb^{sr} was introduced to almost all non-glutinous elite rice cultivars in Hokkaido (12/13 cultivars; **Supplemental Table 2**). The distribution of the *SbeIIb* genotype suggested that there was intensive selection pressure to change the genotype in the Hokkaido breeding population. We propose the following three hypotheses about the cause: (1) *SbeIIb*^{sr} exhibits desirable starch properties in non-glutinous rice varieties; (2) another useful genotype (allele) that is strongly linked is present in this region; (3) it happened by chance. In case 1, *SbeIIb*^{sr} is considered to be the most important allele for controlling starch properties in rice breeding. In case 2, some agricultural traits, in addition to starch properties, may have changed in cultivars with *SbeIIb*^{sr}. Shinada *et al.* (2014) suggests that 'Kitaake' is the key cultivar which was cause of the differentiation of genetic group among the Hokkaido rice population. This genetic group has significant differences in phenotypes such as cold tolerance at the booting stage, amylose content, number of spikelets per panicle compared to other groups. Future studies will reveal whether the candidate regions which identified in this study are relevant for genetic differentiation and phenotypic change. In either case, it is necessary to clarify the effect of *SbeIIb*^{sr} on non-glutinous rice varieties.

SbeIIb^{sr} is a genotype that detected in Hokkaido breeding populations and is expected to be used as breeding material in different cultivation areas where *SbeIIb*^{sr} has not been introduced. Using local breeding varieties as analysis material would make it easy to associate the genotype with the useful phenotype. Local elite cultivars could be cultivated in the same environment, allowing for the observation of slight differences in the phenotype due to genetic differences. As sequence analysis based on next generation sequencing data provides accurate mapping and estimation of candidate genes, there should be no problem with genetic analysis even when using genetically related varieties. Historical studies on unique traits that characterize elite cultivars in each local region and studies on the genetic mechanisms of phenotype would contribute to the development of new cultivars with novel combinations of genes and more desirable agronomic traits.

Author Contribution Statement

Conceived and designed the experiments and wrote the

manuscript: TI and KA. Performed the experiments: TI and KA. Analyzed the data: TI. Received: date; Accepted: date; Published: date

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