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Genetic studies on a starch biosynthesis gene, *sug-h* and the eating quality-related marker in rice (*Oryza sativa* L.)

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YUNJOO LEE

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DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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GENERAL ABSTRACT

Rice is a major cereal crop for supply staple food, then quality improvement of rice regards as important point for production of better food. Rice eating quality has been final goal in rice breeding. Endosperm is major part of rice grain, thus, component and character of endosperm ultimately decide rice eating quality. Several studies have been reported in many countries on rice cooking and eating quality, and starch biosynthesis of endosperm. This report is composed of two parts; one is about identification of starch biosynthesis genes governing sugary endosperm phenotype, the other is genetic study of Koshihikari-specific sequence related to eating quality.

Sugary endosperm, *sugary-1* (*sug-1*), is a mutant trait for starch biosynthesis. The *sug-1* mutant plant produces the severely wrinkled seeds that were accumulated phytoglycogen-like starch. Because of poor seed phenotype which has problems in milling, sugary endosperm rice has not been commercialized despite improved digestibility and enhanced nutritional merits. Two kinds of sugary mutants, *sugary-1* (*sug-1*) and *sugary-h* (*sug-h*) were obtained by chemical mutagenesis from Korean

japonica cultivar, Hwacheong. Grains of the *sug-h* mutant were translucent and amber-colored, and the endosperm appeared less wrinkled than *sug-1*, whereas the soluble sugar content was high. These characteristics provide commercially feasible advantages to the *sug-h* mutant. According to genetic analysis data, the *sug-h* mutant phenotype was controlled by complementary interaction of two recessive genes, *Isoamylase1* (*OsISA1*), which was reported previously, and *Starch branching enzyme IIa* (*OsBEIIa*), which was newly identified in this study. Complementation tests indicated that *OsBEIIa* regulated the properties of sugary endosperm. These results extend our knowledge of the mechanism of starch biosynthesis in rice endosperm, and facilitate the breeding of sugary endosperm rice for better digestibility.

Koshihikari-specific marker, named 'P5' is main target of this study on novel factors related to improvement of rice eating quality. The P5 marker which could be found only in Koshihikari-derived cultivars, was from the report on development of markers for eating quality evaluation. Linkage mapping using two popolations and 72 STS markers indicated that P5 marker was on the long arm of chromosome 11. Two CSSLs were used to confirm the location of P5. *De novo* assembly of Koshihikari was also performed to idenetify Koshihikari-specific full sequence containing P5 region. As a result, this specific seqence was about 70 kb and replaced Nipponbare sequence into Koshihikari-specific sequence. Semi-qRT-PCR results showed that only one gene was expressed among 10 predicted genes in this sequence. The NIL populations were developed and used to identify the effect of this specific sequence on eating quality. Palatability

of NIL lines substitued Koshihikari-specific sequence instead of Samnam (Korean *japonica* cultivar with poor eating quality) was increased than Samnam, indicating that P5 sequence contributed the improvement of rice eating quality. These results will be helpful for elucidating a novel genetic factors for improvement of rice eating quailty by dissection on Koshihikari-specific sequence.

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LIST OF ABBREVIATIONS

BAC	Bacterial artificial chromosome
BSA	Bulked segregant analysis
CSSL	Chromosome segment substitution line
DAF	Day after flowering
dCAPS	Derived cleaved amplified polymorphic sequence
MAS	Marker assisted selection
MNU	N-methyl-N-nitrosourea
ncRNA	Non-coding RNA
PCR	Polymerase chain reaction
RIL	Recombinant inbred line
SNP	Single nucleotide polymorphism
STS	Sequence tagged site
sug-1	sugary-1
sug-h	sugary-h

LITERATURE REVIEW

Composition of rice endosperm starch

Cereal crops accumulate starch in the endosperm as an energy supply. Humans and livestock usually used the starch as the primary carbohydrate component for food or industrial applications. Starch is composed of two kinds of polymers, amylose and amylopectin. Amylose is a linear molecule, joined via α-1,4 linkages, but amylopectin is more complex molecule which contains the glucosyl units, joined via α-1,4 linkages and α-1,6 linkages. Because of semi-crystallinity of amylopectin, starch has insoluble character.

Amylopectin has a tandem-cluster structure which is composed of amorphous and crystalline lamellae, containing many chain branches by α-1,6 linkages. A chains mean no branch amylopectin chains and B chains mean amylopectin chains having branches. B chains are called as three types, B1, B2 and B3 chains, which are connected with one, two and three clusters (Peat et al. 1952). The position and distributed location of amylose and amylopectin still remains unclear. Three types of crystalline structure, A, B and C-type, have been identified based on X-ray diffraction analysis (Hsien-Chih and Sarko 1978; James et al. 2003). These types are distinguished by the density of granule packing and the amount of bound water. The normal starch type in cereal crops is consist of A-type, in which outer double helices structure are arranged with a minimal amount of bound water.

Starch biosynthesis enzymes

Starch is synthesized by four enzyme classes, with multiple subunits in each class: ADP-glucose pyrophosphorylase (AGPase); starch synthase (SS); starch branching enzyme (BE); and starch debranching enzyme (DBE). Other enzymes, such as phosphorylase and disproportionating enzyme, are thought to be involved in starch biosynthesis. BE and DBE have important roles in determining amylopectin structure. BE forms the α-1,6-glycosidic bonds of amylopectin, whereas DBE trims improper branches generated by BE (Fujita 2014). BE in rice grain contains BEI (SBE1), BEIIb (SBE3), and BEIIa (SBE4) isoforms (Dian et al. 2005; Tanaka et al. 2004; Yamanouchi and Nakamura 1992). SSS contains SSS1, SSS2A (SSSII-3), SS2B (SSSII-2), SSS2C (SSSII-1), SSS3A (SSSIII-2), SSS3B (SSSIII-1), and SSS4A (SSSIV-1), SSS4B (SSSIV-2) (Ohdan et al. 2005). DBE contains isoamylase (ISA) and pullulanase (PUL) in rice. ISA has at least three isoforms, ISA1, ISA2, and ISA3, and only one PUL (Fujita et al. 2009).

Reported mutants related to starch biosynthesis in cereal crop

There are several mutants and transgenic plant related to starch biosynthesis in many studies. Previous reports designated BEIIb-deficient mutants in maize and rice as *amylose-extender* (*ae*) mutants, in which the abundance of short amylopectin chains was reduced (Kim et al. 1998; Nishi et al. 2001). Other transgenic research about *BEIIb* gene was reported that the manipulation of BEIIb activity can generate various starch type rice, containing chalky and sugary endosperm (Tanaka et al. 2004). According to Fujita (2014), Isoamylase1 (ISA1)-deficient mutants (*isa1*) were called

as *sugary-1* mutants in rice (*sug-1*) and maize (*su1*). These mutants have a defect in the amylopectin cluster structure, which results in the accumulation of a polymeric water-soluble polysaccharide (WSP) termed phytoglycogen, and a reduction in the starch content (James et al. 2003). There are various *sug-1* mutant types, EM series, reported by Japanese group (Nakamura et al. 1997; Nakamura et al. 1996; Wong et al. 2003). The *sug-1* locus in rice is located on chromosome 8 (Fujita et al. 1999; Yano et al. 1984). In transgenic *sug-1* rice expressing the wheat *ISA1* gene, phytoglycogen synthesis is substantially replaced by starch biosynthesis in the endosperm (Kubo et al. 2005). In maize, double mutant defective in both ISA2 and SSIII generated water-soluble glucans in the mutant endosperm, although single mutants of either ISA2 or SSIII could synthesize normal amylopectin (Lin et al. 2012). Recently, rice novel endosperm mutant, named as *sugary-2*, was reported (Nakagami et al. 2016). Although large granules were shown in the outer region of the *sugary-2* endosperm, the granules in the inner region were smaller. In the intermediate region, both small and large granules coexisted.

Various methods of eating quality evaluation

Several tools have been developed to evaluate the physicochemical properties of the rice grain, including palatability measured by the Toyo taste meter and viscosity profiles measure by the Brabender viscograph procedure and Rapid visco analyzer (RVA) (Bao and Xia 1999; Deffenbaugh and Walker 1989). Evaluation method by Toyo meter is based on hydration retention on the cooked rice grains detected at a given electromagnetic wavelength. The RVA is widely used as a standard tool on rice quality and

industrial manufacturing, simulates the cooking process of rice flour by using specific temperature cycles to reveal the pasting properties of rice starch (Bergman et al. 2004). The other evaluation methods are also used for evaluate the amylose content (AC) by the relative absorbency of iodine stained color in digested solution of rice flour according to (Juliano 1971). In addition, gel consistency (GC), gelatinization temperature (GT), pasting properties (PP), protein content (PC), and texture (Cagampang et al. 1973; Champagne et al. 1999; Juliano et al. 1973; McKenzie and Rutger 1983) are evaluted for eating quality related factors.

Previous reported QTLs and genes associated with grain and cooked rice taste

Several QTLs and genes related to grain and eating quality were reported in rice. Various populations were used for detecting QTLs. Recombinant inbred line (RIL) such as Suweon365/Chucheongbye F_{12-14} (Kwon et al. 2011), Moritawase/Koshihikari F_{7-9} (Wada et al. 2008), Zhenshan97/Delong208 (Wang et al. 2007) and Moritawase/Koshihikari F_{5-6} (Wada et al. 2006), were usually used for QTL mapping. Chromosome segment substitution lines (CSSLs) derived from Asominori/IR24 (Liu et al. 2010) and Koshihikari/Kasalath//Koshihikari (Takeuchi et al. 2007) cross were also used. Double haploid (DH) populations such as CJ06/TN1 (Leng et al. 2014), WYJ2/Zhenshan97B (Tian et al. 2005), and IR64/Azucena cross (Bao et al. 2002) were reported for QTL detection.

Identified genes associated with cooked rice taste are evenly distributed in all chromosomes. *FLO2* (*Floury endosperm 2*) (She et al. 2010), *FLO4* (*Floury endosperm 4*) (Kang et al. 2005) , *RSR1* (*rice starch*

regulator 1) (Fu and Xue 2010), *qPGWC-7* (*percentage of grain with chalkiness*) (Zhou et al. 2009), and *BADH2* (*betaine-aldehyde dehydrogenase 2*) (Chen et al. 2006) were well-known genes. The other genes related to starch biosynthesis also identified as key factors which influence cooked rice taste. These informations of identified genes could use for marker development which was needed in marker assisted breeding program.

CHAPTER I

Isolation of the *sugary-h* endosperm (*sug-h*) gene in rice (*Oryza sativa* L.)

ABSTRACT

Starch biosynthesis is one of the most important pathways that determine both grain quality and yield in rice (*Oryza sativa* L.). Sugary endosperm, *sugary-1* (*sug-1*), is a mutant trait for starch biosynthesis. Plants carrying *sug-1* produce grain that accumulates water-soluble carbohydrates instead of starch, even after maturity. Although this trait confers improved digestibility and enhanced nutritional merits, sugary endosperm rice has not been commercialized due to the severely wrinkled grains and subsequent problems in milling. We performed chemical mutagenesis on the Korean *japonica* cultivar Hwacheong, and identified a mild sugary mutant, *sugary-h* (*sug-h*). Grains of the *sug-h* mutant were translucent and amber-colored, and the endosperm appeared less wrinkled than *sug-1*, whereas the soluble sugar content was high. These characteristics confer greater marketability to the *sug-h* mutant. Genetic analyses indicated that the *sug-h* mutant phenotype was controlled by complementary interaction of two recessive genes, *Isoamylase1* (*OsISA1*), which was reported previously, and *Starch branching enzyme IIa* (*OsBEIIa*), which was newly identified in this study. Complementation tests indicated that *OsBEIIa* regulated the properties of sugary endosperm. These results

extend our knowledge of the mechanism of starch biosynthesis in rice endosperm, and facilitate the breeding of sugary endosperm rice for better digestibility.

Key words: Map-based cloning, *OsBEIIa*, *OsISA1*, rice, *sugary-h*, sugary endosperm

INTRODUCTION

Rice is the staple food for more than 3 billion people globally. The endosperm is an edible part of the rice grain, and has been one of the major targets for grain geneticists and breeders to enhance grain yield and quality. Endosperm development directly regulates grain formation at the grain filling stage. Mature rice endosperm contains starch, storage proteins, lipids, and other substances. Studies on starch have been an essential focus in rice research. Starch is the primary component that makes cereal crops economically and commercially important. Starch research is also becoming increasingly relevant for industrial and manufacturing applications.

Rice starch is composed of amylose (linear α-1,4-polyglucans) and amylopectin (α-1,6- branched polyglucans). Amylopectin has a distinct fine structure called multiple cluster structure, and accounts for approximately 65–85% of storage starch (Nakamura 2002). Starch is synthesized by four enzyme classes, with multiple subunits in each class: ADP-glucose pyrophosphorylase (AGPase); starch synthase (SS); starch branching enzyme (BE); and starch debranching enzyme (DBE). Other enzymes, such as phosphorylase and disproportionating enzyme, are thought to be involved in starch biosynthesis. BE and DBE have important roles in determining amylopectin structure. BE forms the α-1,6-glycosidic bonds of amylopectin, whereas DBE trims improper branches generated by BE (Fujita 2014).

BE isoforms are classified into two groups, BEI (RBE1) and BEII. Cereals have two BEII isozymes, BEIIa (RBE4) and BEIIb (RBE3). These isoforms

are classified according to the transferred amylopectin chain length. For example, BEII transfers shorter chains than BEI, and BEIIb transfers shorter chains than BEIIa, during extended incubations (Mizuno et al. 2001). The expression patterns of BE isoforms also differ. BEI and BEIIa transcripts have been localized in the endosperm and other tissues, whereas BEIIb is expressed only in the endosperm and reproductive tissues. In rice, BEIIa is expressed earlier than either BEIIb or BEI (Mizuno et al. 2001; Ohdan et al. 2005). Previous reports designated BEIIb-deficient mutants in maize and rice as *amylose-extender* (*ae*) mutants, in which the abundance of short amylopectin chains was reduced (Kim et al. 1998; Nishi et al. 2001). Other transgenic research about *BEIIb* gene was reported that the manipulation of BEIIb activity can generate various starch type rice, containing chalky and sugary endosperm (Tanaka et al. 2004). The RNA interference results demonstrated that reduced expression of BEIIa (SBEIIa) caused increase of amylose content in wheat endosperm (Regina et al. 2006). However, the specific functional role of BEIIa has not been elucidated in rice because the seed phenotypes of BEIIa-deficient mutants and wild-type plants are not significantly different (Fujita 2014).

DBEs directly hydrolyze α-1,6-glycosidic linkages of α-polyglucans. DEBs are classified into two types in higher plants, Isoamylase (ISA1, ISA2, and ISA3) and Pullulanase (PUL). According to Fujita (2014), Isoamylase1 (ISA1)-deficient mutants (*isa1*) were called as *sugary-1* mutants in rice (*sug-1*) and maize (*su1*). These mutants have a defect in the amylopectin cluster structure, which results in the accumulation of a polymeric water-soluble polysaccharide (WSP) termed phytoglycogen, and a reduction in the starch content (James et al. 2003). There are various *sug-1* mutant

types, EM series, reported by Japanese group (Nakamura et al. 1997; Nakamura et al. 1996; Wong et al. 2003). The *sug-1* locus in rice is located on chromosome 8 (Fujita et al. 1999; Yano et al. 1984). In transgenic *sug-1* rice expressing the wheat *ISA1* gene, phytoglycogen synthesis is substantially replaced by starch biosynthesis in the endosperm (Kubo et al. 2005). This result implies that ISA1 is essential for amylopectin crystallinity and biosynthesis in both rice and wheat. In maize, double mutant defective in both ISA2 and SSIII generated water-soluble glucans in the mutant endosperm, although single mutants of either ISA2 or SSIII could synthesize normal amylopectin (Lin et al. 2012). By contrast, the contribution of PUL for amylopectin trimming was much smaller than that of ISA1, and PUL function partially overlaps with that of ISA1 (Fujita et al. 2009).

Sugary endosperm mutants have great potential for rice breeding because of their desirable grain properties. Grains of *sug-1* mutants primarily contain water-soluble carbohydrates instead of starch, even after maturity. This results in improved grain digestibility; therefore, these mutants have good potential value for breeding programs and commercial uses. However, sugary endosperm mutants have not been used in practice due to the severely wrinkled grains and subsequent problems in milling. We developed a mild-type sugary mutant in rice, *sugary-h* (*sug-h*), which displayed an intermediate phenotype between the *sug-1* mutant and wild type. Grains of the *sug-h* mutant have better quality for subsequent processing and higher yield than *sug-1*. In addition, palatability, protein and amylose content which are crucial for breeding were increased in *sug-h* mutant (Yoon et al. 2009). Therefore, *sug-h* mutants could be valuable

for practical applications and nutritional aspects. This study performed map-based cloning to identify the genes responsible for the *sug-h* phenotype. Our results provide new insights into starch biosynthesis, and identify a potential resource for utilizing the sugary endosperm mutation for commercial benefit.

MATERIALS AND METHODS

Plant materials

The *sug-h* mutant was induced by N-methyl-N-nitrosourea (MNU) treatment on the Korean *japonica* cultivar Hwacheong (Koh and Heu 1994). The F_2 population was derived from a cross between the *sug-h* mutant (*japonica*) and Milyang 23 (M.23, a Korean *tongil*-type cultivar). F_3 seeds were classified into three groups: normal, sugary, and heterozygous type. Two F_2 individuals that displayed the sugary type in F_3 seeds were developed for the F_3 population. To calculate the segregation ratio, another F_2 population derived from a cross between the *sug-h* mutant and wild-type Hwacheong was used. The *sug-1* mutant line was selected from the *sug-h*/Hwacheong F_2 population, and isolated to the F_6 generation via self-pollination. Normal-type seeds were designated as 'N-type' and sugary-type seeds were designated as 'S-type'. S-type seeds were grouped into 'I-type' (*sugary-1* type), mixed-type (I-type and II-type), and 'II-type' (*sugary-h* type) in segregating populations. These populations were cultivated using conventional methods at the Experimental Farm of Seoul National University.

Phenotypic analysis

All F_1, F_2, and F_3 seeds were dehulled and observed under a microscope. S-type and N-type seeds were distinguished by seed thickness and severity of wrinkling. All seeds from a whole panicle were used for phenotyping to minimize phenotypic error resulting from differences in seed maturity. Grain dimensions including length, width, and thickness

were measured using digimatic calipers (Mitutoyo, Japan) and analyzed using the IBM SPSS statistics program. Each seed was stained with iodine solution [0.1% (w/v) I_2 and 1% (w/v) KI] to detect starch.

Scanning electron microscopy

Seeds and starch samples were visualized with a scanning electron microscope (SEM) according to the previously published method of Fujita *et al.* (2003). Gold-coated seeds and starch samples were observed using a SUPRA 55VP Scanning Electron Microscope (Carl Zeiss, Germany).

X-ray diffraction analysis of starch

One of the samples for morphological analysis of starch properties, *Sug-1/sug-h*, possessed normal *OsISA1* and mutated *osbe2a* alleles. X-ray diffraction was used to determine the structures of starch according to the methods described previously by Kubo *et al.* (2005). The X-ray diffraction patterns of isolated insoluble glucans were obtained with a copper (nickel foil-filtered) K_α radiation using D8 Advanced X-ray diffractometry (Bruker, Germany) at 40 kv and 40 mA. The scanning region of the two-theta angle (2θ) ranged from 4.0 to 40.0° with a scan speed of 0.5 deg min^{-1}.

Chain-length distribution of amylopectin by HEAEC-PAD

The chain-length distributions of α-glucans from wild-type and mutants endosperm were analyzed using the capillary electrophoresis methods by high performance anion-exchange chromatography with pulsed amperometric detection (HEAEC-PAD) in previous reports (Hanashiro et al. 1996; Yoon et al. 2012).

DNA extraction and PCR amplification

Total genomic DNA was extracted from young leaves of F_2, F_3 plants and their parents according to the method of McCouch *et al.* (1988) with modifications. Polymerase chain reactions (PCR) were performed in a reaction volume of 20 μl containing 100 ng of template DNA, 0.1 μM each primer, 2.5 mM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 0.5 U Taq DNA polymerase. PCR amplification was carried out in a DNA Engine Tetrad 2 and Dyad Thermal Cycler (Bio-Rad, USA) using the following reaction conditions: 5 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 30 s at 56 °C, and 40 s at 72 °C; and 10 min at 72 °C for final extension. PCR products were separated on 2.5% (w/v) agarose gels containing 0.15 μg ml^{-1} ethidium bromide (EtBr) in 0.5 × TBE buffer.

Linkage mapping of the *sug-h* mutant

We performed bulked segregant analysis (BSA) to genetically map and isolate genes related to the *sug-h* mutant (Michelmore et al. 1991). Ten N-type plants, ten heterozygote-type plants (N-type and S-type), and ten S-type plants were selected from the *sug-h*/M.23 F_2 population for the first BSA to identify gene distinguishing N-type and S-type. For the second BSA, ten I-type plants, 12 mixed-type plants (I-type and II-type), and 12 II-type plants were selected from the *sug-h*/M.23 F_3 population, in which the sugary allele was fixed. Three bulked samples containing randomly combined equal amounts of DNA were used for genotyping. Then, fine mapping was conducted on two chromosomes with flanking sequence tagged site (STS) markers, which were developed by designing primers

based on the DNA sequence differences between *indica* and *japonica* rice cultivars (Chin et al. 2007). Additional STS and derived cleaved amplified polymorphic sequence (dCAPS) primers were designed with Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3) for additional fine mapping based on the available rice genome sequence data (http://www.gramene.org, http://rgp.dna.affrc.go.jp/IRGSP/, http://www.ncbi.nlm.nih.gov). Primer sequences and other information for DNA markers designed and used in this study are listed in Table 1-1.

Candidate gene analysis

To validate the candidate gene models, full-length genomic DNA of each candidate gene in Hwacheong and the *sug-h* mutant was amplified by PCR. PCR products were purified using the PCR purification kit (Inclone, Korea) and transformed into *E. coli* strain DH5α, followed by ligation of PCR amplicons into the pGEM-T Easy Vector (Promega, USA). Transformed plasmid sequences were analyzed with an ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA). The PCR clones were verified by sequence alignment with the original parent using CodonCode Aligner software (CodonCode Corporation, USA). Based on the results of the sequencing analysis, multiple alignments were performed using a public database (http://www.ch.embnet.org/software/BOX_form.html).

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from various tissues of wild-type, mutant, and transgenic plants using MG Total RNA Extraction kit (Doctor Protein, Korea). The RNAs were converted into first-strand cDNA using M-MLV

Reverse Transcriptase (Promega, USA). Quantitative RT-PCR was performed using SYBR Premix ExTaq (Takara Bio, Japan) according to the manufacturer's instructions. Gene expression levels were evaluated in leaf, stem, and root samples collected at maximum tillering stage and seed samples collected at 5, 10, and 20 days after flowering (DAF). The following gene-specific primer sets were used: *OsISA1*-RT, 5′-CAAATGCGCAATTTCTTTGTT-3′ (sense) and 5′-GTCCCAGCGGAAATAATTGA-3′ (antisense); *OsBEIIa*-RT, 5′-GCCAATGCCAGGAAGATGA-3′ (sense) and 5′-GCGCAACATAGGATGGGTTT-3′ (antisense) (Zhang et al. 2012); control *UBQ*-qPCR, 5′-GAGCCTCTGTTCGTCAAGTA-3′ and 5′-ACTCGATGGTCCATTAAACC-3′ (Tanaka et al. 2011). Quantitative RT-PCR was performed using a C1000 thermal cycler (Bio-Rad, USA).

Table 1-1 Molecular markers used for fine mapping of the *sug-h* mutant

Marker	Chr.	Type	Forward primer (5'-3')	Reverse primer (5'-3')
S08105	8	STS	CCGTGCATATAGAGGAAAACG	ACACTCACACGTCATGAGCA
S08106	8	STS	TTACGGATTGTCACGGTTTT	GGAATTTGTCACTGGTTTCCA
S08107	8	STS	TTGGTAATGCCCATGCTAGA	CACGATTCGGTCATTTCAGA
S04056A	4	STS	CTGATTGCTCCCCTGAAGAG	TGCCTGCACTTGAAATCAGA
S04056B	4	STS	CCCTTGGATCGAATCGCTTC	CACGTCCTTCGGCGATTCAG
S04056C	4	STS	GTTGTTTCTCGTGAGGTGTTAT	AATTTCACACTGTGGTTGTTTA
AL731A	4	dCAPS/*Hha*I	GAATGCCCCTTAGGGTCAAAAGCTTTTG**C**G	AGATGAGATGCCCTGACCAAAT
AL731B	4	dCAPS/*Taq*I	ATAGCGTTGGTGTTTAGTACAGCTTA**T**C	CATGTGTCCTAGAAGAGTGCAA
S04057	4	STS	GCGTCAGCGGCGCATTATCC	CAGTAGCTGACCGTCTCACG
S04058	4	STS	GATCCATGCAGTTGATTGTGA	TCGTCTTATCTAAAAAGAAAATTTGA

Complementation of the *sug-h* mutant

The RNA interference (RNAi) vector was constructed by PCR amplification of a 291 bp fragment from *OsISA1* and a 206 bp fragment from *OsBEIIa* cloned from Hwacheong cDNA. These fragments were subcloned into pDONR201 (Invitrogen, USA), and then transferred into the RNAi vector pH7GWIWG(II) using Gateway BP and LR clonase enzyme mixes (Invitrogen, USA). The full-length *OsBEIIa* cDNA was amplified from Hwacheong cDNA and used for constructing the overexpression vector. The amplified fragment was transferred into pMDC32 via pCR™ 8/GW/TOPO® TA Cloning Kit (Invitrogen, USA). The RNAi constructs were transformed into wild-type Dongjin (a *japonica* cultivar), and the overexpression construct was transformed into callus of the *sug-h* mutant. Transformation was performed using a modification of the previously published *Agrobacterium*-mediated transformation method (Nishimura et al. 2006).

Histological GUS assay

The 1,909 bp region upstream from the start codon of *OsBEIIa* was amplified and cloned into the binary vector pHGWFS7 using Gateway BP and LR clonase enzyme mixes (Invitrogen, USA). The final construct was introduced into wild-type Dongjin by *Agrobacterium*-mediated transformation. Transgenic plants containing the *OsBEIIa* promoter::GUS reporter construct were selected, and T_0 plants were used for GUS assays. GUS staining was performed as described previously (Jefferson et al. 1987). X-Gluc buffer solution was vacuum-infiltrated into several different tissue samples. The samples were incubated overnight in X-Gluc buffer solution

at 37 ℃, and then washed with a graded ethanol series.

Native-PAGE/activity staining of DBE and BE

Crude enzyme was extracted using the method described by Yamanouchi and Nakamura (1992) from ten seeds at late-milky stage. Native-PAGE/activity staining of DBE was performed using the modified method of Nakamura et al. (1997). Native slab gel was prepared with 6.5% (w/v; resolving gel) containing 0.3% (w/v) potato tuber amylopectin (Sigma, USA), and 3.3% (w/v; stacking gel) acrylamide. Electrophoresis was carried out at 4 ℃ at constant current of 20 mA for 2.5 h. For detection of the isoamylase activity, the gel was rinsed with 20 ml of 50 mM Na-acetate (pH 5.4), 50 mM 2-mercaptoethanol and 50 mM $CaCl_2$ at room temperature and then incubated at 37 ℃ for 3 h with 20 ml of the same buffer solution. Native-PAGE/activity staining of BE was carried out according to the modified method by Yamanouchi and Nakamura (1992). A slab gel prepared with 5% (w/v; resolving gel) and 3.3% (w/v; stacking gel) acrylamide. Electrophoresis was carried out at 4℃ at constant current of 20 mA. After electrophoresis, the gel was imbibed with 20 mL of a solution containing 50 mM HEPES-NaOH buffer (pH 7.4) and 20% (v/v) glycerol for 15 min on ice. For detection of BE, the gel was incubated in 20 ml of the reaction mixture, which consisted of 50 mM HEPES-NaOH buffer (pH 7.4), 50 mM Glc-1-P (Sigma, USA), 2.5 mM AMP (Sigma, USA), 10% (v/v) glycerol, and rabbit muscle phosphorylase a (about 30 units; Sigma, USA) for 6 h at 30℃ with gentle shaking. Iodine solution [0.1% (w/v) I_2 and 1% (w/v) KI] was used for staining both gels.

RESULTS

Phenotypic characterization of the *sug-h* mutant

The *sug-h* mutant did not exhibit abnormal phenotypes during the vegetative stage of plant growth, although the heading date was delayed and plant height was reduced compared with that of wild-type plants (Table 1-2; Fig. 1-1). Grains of the *sug-h* mutant displayed an intermediate phenotype between the wild-type and *sug-1* mutant grains, that was medium thickness and slightly wrinkled (Fig. 1-2a–f). Wild type, *sug-1*, and *sug-h* mutants showed significant differences in grain dimensions and thickness. The grain length and width of *sug-h* mutant was slightly longer and shorter, respectively, than that of wild-type (Table 1-2).

Figure. 1-1 Plant and panicle morphology of wild-type rice (Hwacheong) and mutants. **(a–b)** Plant phenotype of wild-type and mutant plants 54 days after transplanting (a) and at the milky stage (b). **(c)** Panicle length of wild-type and mutant plants at the yellow ripe stage.

Table 1-2 Agronomical characters and dimensions of wild-type and mutants grain

Trait	HD	CL (cm)	PN	SN	TGW (g)	GL (mm)	GW (mm)	GT (mm)	GS
WT	Aug. 17	85.9±1.6[a]	14.6±1.4[b]	98.9±17.0	19.9±0.2[a]	5.1±0.1[b]	3.1±0.1[b]	2.17±0.03[c]	1.67±0.54[a]
sug-1	Aug. 20	87.9±3.0[b]	11.1±3.6[a]	90.3±10.9	8.8±0.3[b]	4.9±0.1[a]	3.0±0.1[a]	1.07±0.13[a]	1.65±0.05[a]
sug-h	Aug. 20	75.6±1.3[c]	16.4±1.8[b]	101.8±9.8	14.5±0.2[c]	5.4±0.1[c]	2.9±0.1[a]	1.70±0.11[b]	1.86±0.07[b]

HD, heading date; CL, culm length; PN, number of panicles per plant; SN, number of spikelets per panicle; TGW, 1,000-grain weight for brown rice; GL, grain length; GW, grain width; GT, grain thickness; GS, grain shape (length/width); WT, wild type (Hwacheong); *sug-1*, *sugary-1*; *sug-h*, *sugary-h*.

[*] Different letters denote significant differences. Ten biological replicates were used to measure for each of the traits

Morphological properties of starch in mutant endosperm

Grains were stained with iodine to identify starch components. Phytoglycogen, which does not stain with iodine, had the highest abundance in *sug-1* endosperm. The *sug-h* mutant grain was partially stained in outermost endosperm layers, whereas the entire wild-type endosperm was stained (Fig. 1-2g–i). These results indicate that the endosperm starch components in both sugary mutants differed from those in the wild type, and starch production was at least partially restored in the *sug-h* endosperm.

Cross sections of polished rice grains were observed with SEM. The images revealed that *sug-1* and *sug-h* mutants had loosely packed, abnormal starch granules in the cutting plane compared with the densely packed granules in wild-type polished grains. The *sug-h* endosperm had starch granule packing that was intermediate between that of *sug-1* and the wild type (Fig. 1-2j-o). Starch granules in *sug-1* and *sug-h* mutants also displayed irregular shapes with round edges, whereas those in the wild type displayed polygonal shapes with sharp edges (Fig. 1-2p–r). These results indicate that aberrant starch production was involved in the abnormal phenotypes of *sug-1* and *sug-h* mutants.

Starch crystallinity was investigated by performing X-ray diffraction analysis of endosperm starch from the wild type, *sug-1*, *sug-h*, and *Sug-1*/*sug-h*. Diffraction patterns of *sug-1* and *sug-h* starch displayed lower peak intensity than that of the wild type (Fig. 1-3). These X-ray diffraction patterns indicate that the crystallization of *sug-1* and *sug-h* starch was lower than that of wild-type starch, as reported by Yoon et al. (2009).

Starch from *sug-h* mutant had an intermediate crystallization level between that from *sug-1* and the wild type. However, *Sug-1*/*sug-h*, which has a normal phenotype, displayed similar starch crystallinity to that of wild-type starch. These results indicate that *osbe2a* did not affect the starch structure and crystallization in the presence of normal *OsISA1*.

Amylopectin fine structure in mature mutant endosperm

Fine structural feature of amylopectin in wild-type and mutant endosperms were compared in detail by analysis of amylopectin chain-length distribution using HEAEC-PAD. Both sugary mutants had the definitely increased amount of short chains in the range of DP 6-10 (Fig. 1-4a-d). This result was similar to the previous reports (Nakamura et al. 1997; Wong et al. 2003; Yoon et al. 2009), indicating that mutation on ISA altered the fine structure of amylopectin into phytoglycogen in rice endosperm. The difference between *sug-1* and *sug-h* on fine structure of amylopectin was relative amount of short chains. Most range of chain-length distribution of amylopectin in *sug-h* was similar with that of *sug-1*, except in the range of DP 7-8 and DP 13-17 (Fig. 1-4e-f). Structural feature of amylopectin suggest that the *sug-h* phenotype was determined by the relative amount of short chains.

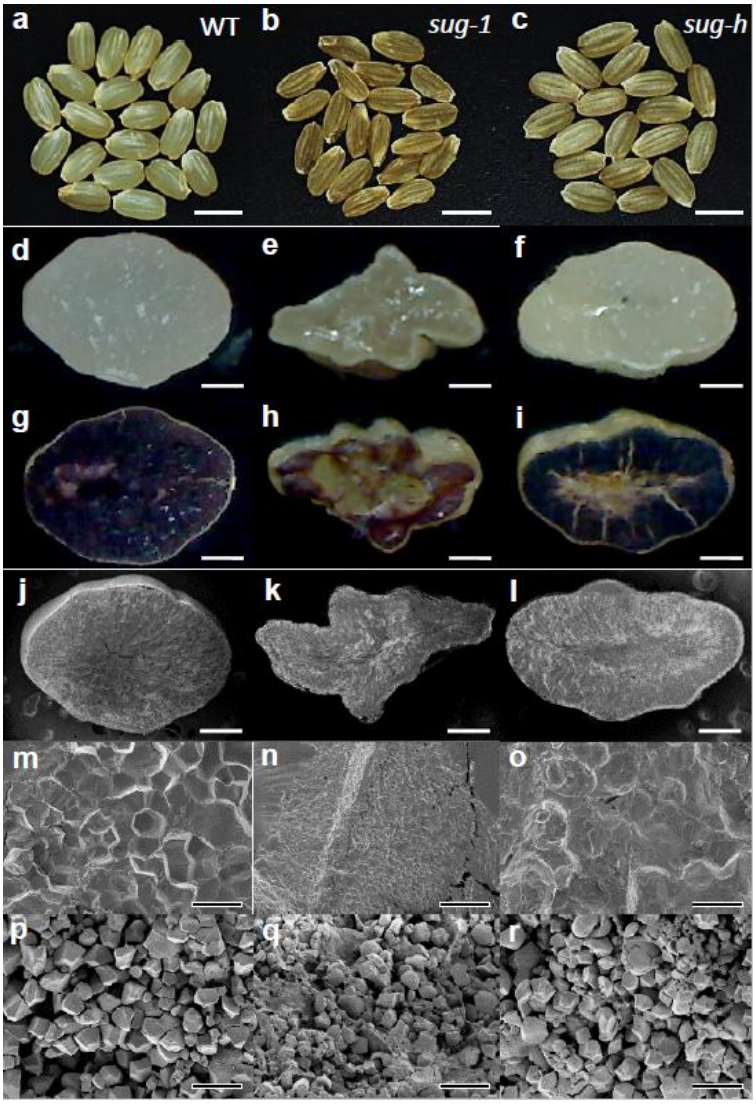

Figure. 1-2 Optical and scanning electron microscopy observation of grain phenotype and starch granule structure in wild-type rice (Hwacheong) and *sug-1* and *sug-h* mutants. **(a–c)** The *sug-h* mutant grain exhibits a phenotype that is intermediate between that of the wild type and *sug-1*. Bars=4 mm. **(d–i)** Cross sections of wild-type, *sug-1*, and *sug-h* kernels at the mature stage. Sectioned seeds were stained with iodine solution. Bars=0.5 mm. **(j–o)** SEM observations of wild-type, *sug-1*, and *sug-h* mutant endosperm. (j–l) Bars=0.5 mm. (m–o) Bars=10 μm. **(p–r)** SEM observations of starch granule structures in wild-type, *sug-1*, and *sug-h* mutant endosperm. Bars=10 μm.

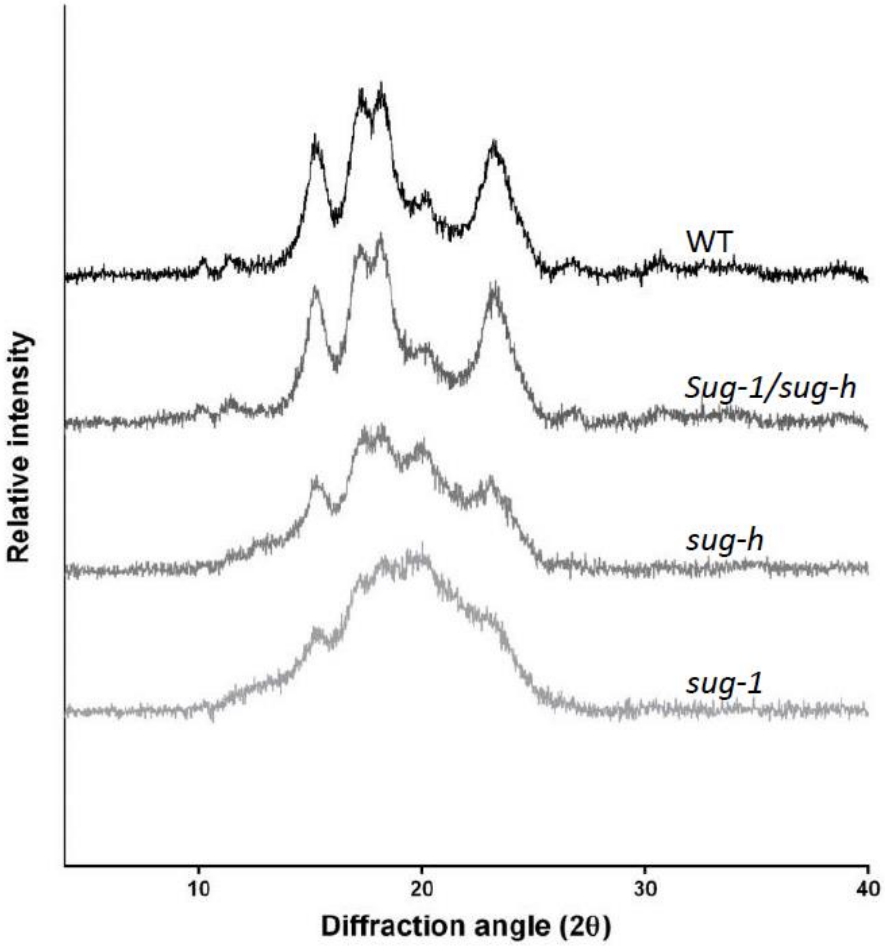

Figure. 1-3 X-ray diffraction analysis of purified starch granules from mature endosperm of wild-type rice (Hwacheong), *sug-1*, *sug-h*, and *Sug-1*/*sug-h.* The seeds showed a normal *OsISA1* and a mutated *osbe2a* genotypes were designated as *Sug-1*/*sug-h*.

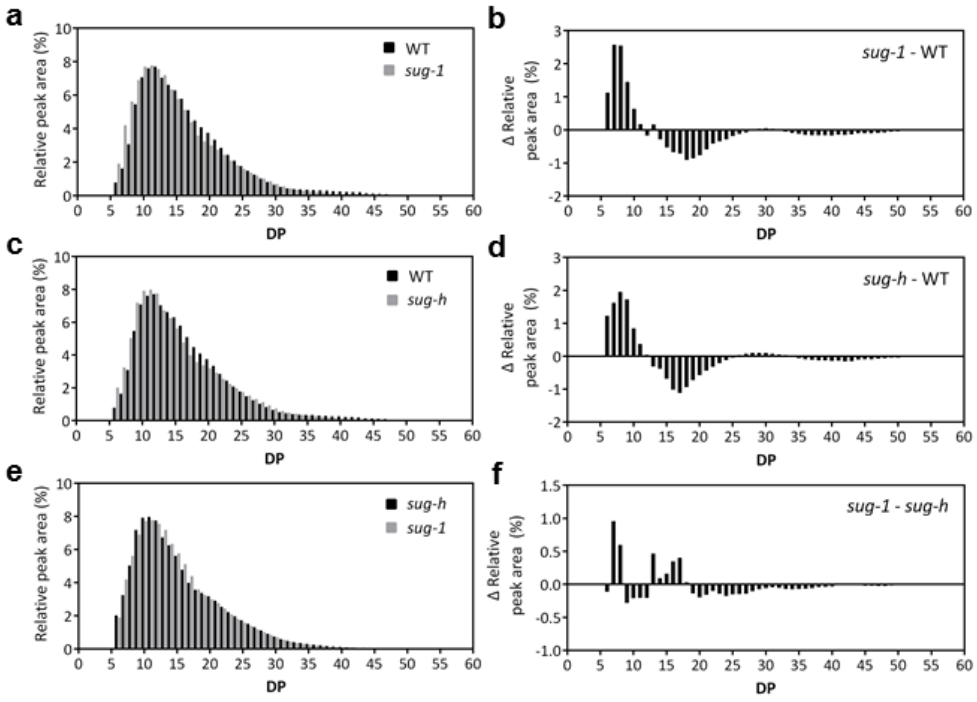

Figure. 1-4 Comparison of chain length distribution of amylopectin in rice endosperm between wild type (Hwacheong) and mutant. The left panels showed chain-length profiles, and the right panels showed differences in chain-length profiles. The number of individual chains was represented as a percentage of the total branch chains of DP ≤60 on the molar basis. Chain-length profiles and differences between WT and *sug-1* mutant **(a–b)**, between WT and *sug-h* mutant **(c–d)**, and between *sug-1* and *sug-h* mutant **(e–f)**.

Map-based cloning of genes related to the *sug-h* phenotype

We performed map-based cloning to elucidate the genes related to the *sug-h* phenotype. Normal-type seeds were designated as 'N-type' and sugary-type seeds were designated as 'S-type'. S-type seeds were grouped into 'I-type' (*sugary-1* type), mixed-type (I-type and II-type), and 'II-type' (*sugary-h* type) in segregated populations. F_2 seeds derived from a cross between the *sug-h* mutant and Hwacheong were used for segregation ratio analysis. The results from 352 F_2 seeds identified 271 N-type, 57 I-type, and 24 II-type seeds, which fit the expected ratio of 12:3:1 (P=0.45) (Table 1-3). Expected genotypes of N-type, I-type and II-type will be *Sug-1_ Sug-h_* , *sug-1sug-1Sug-h_* , and *sug-1sug-1sug-h sug-h*, respectively. Based on this result, we hypothesized that the *sug-h* phenotype was controlled by the epistatic interaction between two genes. One of these genes is the preceding gene, which is related to the sugary endosperm phenotype, and the other is the interacting gene, which reduces the severity of the sugary abnormality in starch biosynthesis.

Preliminary mapping was initially conducted on 352 F_2 plants derived from *sug-h*/M.23, and mapped the first gene related to the sugary phenotype. Bulked DNA for the first BSA was prepared to distinguish N-type, heterozygote-type (N-type and S-type), and S-type plants in the F_2 population. Two of seventy-two STS markers across all chromosomes, S08105 and S08107 on chromosome 8, were used as the flanking markers for fine mapping (Fig. 1-5a). Eighteen genes were identified in the fine-mapped region. The *OsISA1* gene sequence (Os08g40930) of Hwacheong and *sug-h* mutant was primarily compared, because previous studies

reported that *OsISA1* affected the sugary phenotype in *sug-1* rice (Kubo et al. 1999; Nakamura et al. 1992; Nakamura et al. 1996). Our sequence analysis revealed that nucleotide residue 6,179 in *OsISA1*, which was adenine (A) in the wild type, was substituted with guanine (G) in *sug-1* and *sug-h* mutants, thereby changing the amino acid from glycine (Gly) to aspartic acid (Asp). These results indicate that the sugary endosperm phenotype could be caused by a point mutation on *OsISA1*, which was shared by both *sug-1* and *sug-h* mutants.

One of the objectives in this study was to identify the second gene controlling the thickness and wrinkling of the sugary endosperm. Therefore, 837 F_3 plants were derived from two individual F_2 plants in which the *osisa1* allele was fixed, for efficient mapping. Bulked DNA for the second BSA was prepared from I-type, mixed-type (I-type and II-type), and II-type plants selected from the F_3 population. The second BSA revealed that markers and the *sug-h* phenotype co-segregated in two loci on chromosome 8 and chromosome 4, indicating that the second gene was located on chromosome 4. For fine mapping, S04056A and S04058 were selected as flanking markers. One STS marker, S04057, and two dCAPS markers, AL731A and AL731B within the AL731641 clone, were additionally designed and applied for linkage analysis. These results reduced the size of the candidate region to 12.9 kb, which contained the following three genes: *1,4-α-glucan-branching enzyme 2*; *Ser/Thr protein phosphatase family protein*; and *histone deacetylase* (Fig. 1-5b). These genes were sequenced and compared in Hwacheong and *sug-h* mutant, and a point mutation was detected in the *1,4-α-glucan-branching enzyme 2* (*OsBEIIa*; *OsSBE4*) gene of the *sug-h* mutant. We found that the nucleotide

residue 4,732 in wild-type *OsBEIIa*, which was G, was substituted with A in the *sug-h* mutant, thereby changing the amino acid from Gly to Asp. This amino acid substitution was not found in other grain species including maize, sorghum, barley, and wheat (Fig. 1-6). These combined results indicate that the *sug-h* mutant might be caused by single point mutations resulting in amino acid substitutions in both *OsISA1* and *OsBEIIa*.

Table 1-3 Genetic analysis of the *sug-h* mutant using F_3 seeds

Cross combination	No. of seeds				$\chi^2_{0.05}$* (12:3:1)	*P* value
	N-type	S-type		Total		
		I	II			
Hwacheong/*sug-h*	271	57	24	352	1.595	0.451
***sug-h*/Hwacheong**	152	28	11	191	2.312	0.315

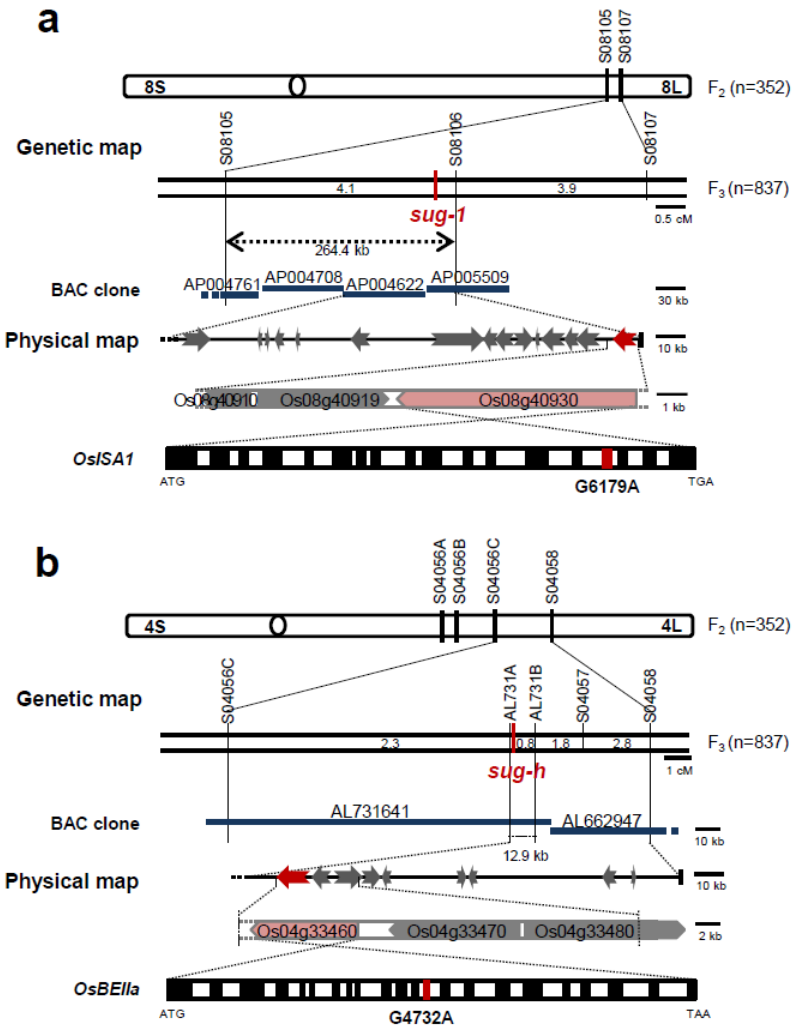

Figure. 1-5 Map-based cloning of the *sug-h* mutant. **(a)** Candidate gene of the first bulked segregant analysis, Os08g40930, was located on the AP005509 BAC clone and contained 18 exons (black boxes) and 17 introns (white boxes). A point mutation, G to A, occurred in the 15th exon (red box). **(b)** Schematic representation of *OsBEIIa* on the long arm of chromosome 4. Candidate region of the gene related to the *sug-h* mutant was located within the AL731641 clone. Schematic structure of the candidate gene, Os04g33460, contained 22 exons (black boxes) and 21 introns (white boxes). A point mutation, G to A, occurred in the 13th exon (red box). Gray arrows indicate the main genes with known functions within the candidate region. Red arrow indicates the candidate gene. ATG and TAA indicate the initiation and termination codons, respectively.

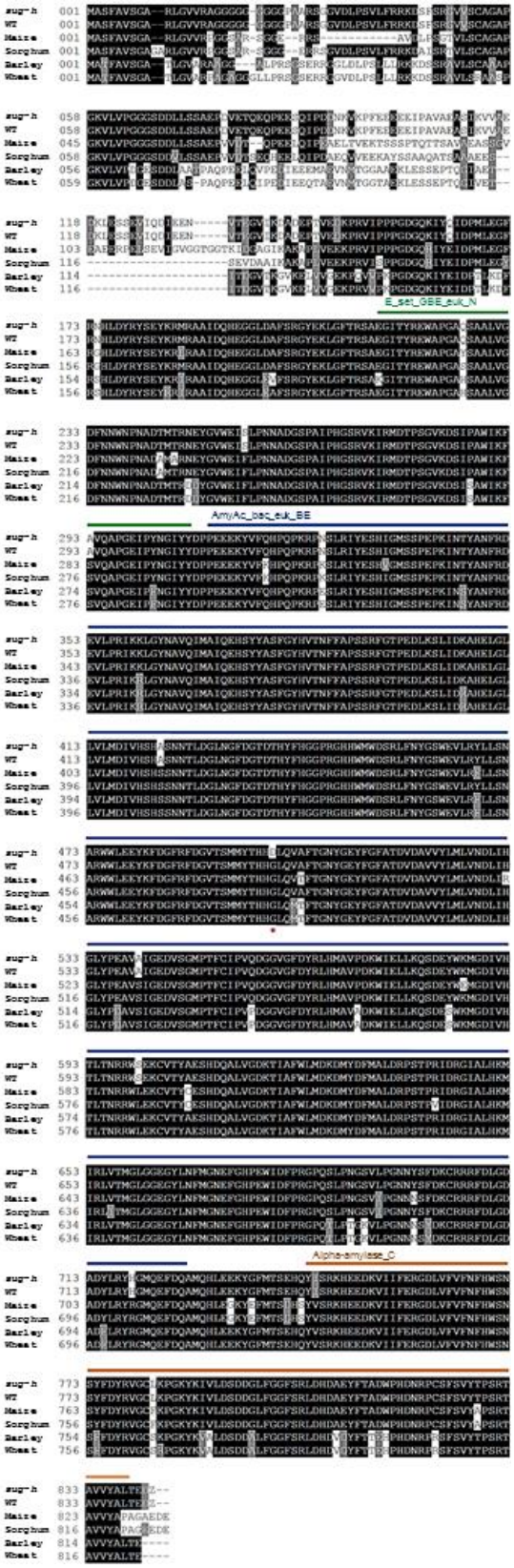

Figure. 1-6 Multiple alignments of cereal OsBEIIa proteins. Protein of the *sug-h* mutant (top line) was aligned with that of wild-type rice (Hwacheong) and four cereal plants (barley, maize, sorghum, and wheat). Black boxes indicate identical residues; gray boxes indicate similar residues. Mutated region is marked with an asterisk. Color bars indicate the domains; E_set_GBE_euk_N (green), AmyAc_bac_euk_BE (blue), and Alpha-amylase_C (orange).

Transgenic complementation of the *sug-h* mutation

To confirm the function of *OsBEIIa* in the *sug-h* mutant, we generated dsRNA-mediated interference (RNAi) transgenic plants. T_1 seeds of the *OsBEIIa*-RNAi transgenic line had normal phenotype, suggesting that a single mutation of *OsBEIIa* had no effect on seed phenotype (Fig. 1-7a). To evaluate interactions between *OsISA1* and *OsBEIIa*, artificial crossing was performed between the *OsBEIIa*-RNAi T_0 plant and the *sug-h* mutant. After phenotypic selection, F_2 seeds from the artificial cross were planted for co-segregation analysis of phenotype, genotype, and RNA expression. Phenotypes of segregated F_2 seeds showed that N-type seeds were slightly thinner than wild-type (Dongjin) seeds. By contrast, the thickness of I-type and II-type seeds was not significantly different from that of *sug-1* and *sug-h* seeds, respectively (Fig. 1-7b). Genotype analysis using an antibiotic resistance gene-specific primer showed that PCR bands were produced in all II-type plants, but no bands were amplified in all I-type plants (data not shown). The qRT-PCR analysis indicated that the relative *OsBEIIa* expression levels were higher in each F_2 plant derived from I-type seed than in the *sug-h* mutant (Fig. 1-7c), whereas the relative *OsBEIIa* expression levels in each F_2 plant derived from II-type seed were lower than that in the *sug-h* mutant (Fig. 1-7d). These results show that phenotype, genotype, and RNA expression co-segregate in the *OsBEIIa*-RNAi-4/*sug-h* F_2 population, indicating that the two genes are associated with the *sug-h* phenotype.

We also generated transgenic plants overexpressing *OsBEIIa*, in which a vector was introduced into the *sug-h* mutant to complement the

phenotype. According to the mapping results that the *sug-h* mutant might be caused by single point mutations in both *OsISA1* and *OsBEIIa* genes, this complementation test was intended to reconstruct the *sug-1* phenotype from *sug-h* mutant. Most T_1 seeds of 35S::*OsBEIIa*-11 T_0 plants were thinner and more wrinkled than *sug-h* seeds (Fig. 1-7a, e). The qRT-PCR analysis indicated that *OsBEIIa* transcript levels were higher in transgenic T_1 plants than in *sug-h* mutant (Fig. 1-7f). These complementation data suggest that *osbe2a* may improve the *sug-h* phenotype by producing grains that are less wrinkled.

Gene expression patterns of *OsBEIIa* and *OsISA1*

To investigate the expression patterns of *OsISA1* and *OsBEIIa* in different organs, we performed qRT-PCR analyses and β-glucuronidase (GUS) reporter gene assay. The qRT-PCR analyses showed that *OsBEIIa* was expressed primarily in leaf, stem, and seed in wild-type and *sug-h* mutant plants (Fig. 1-8a). *OsBEIIa* expression in 10 DAF seeds was slightly down-regulated in *sug-h* mutant compared with that in the wild type, although this change was not statistically significant. By contrast, *OsISA1* expression in *sug-h* seeds was significantly lower than that of the wild type, and *OsISA1* expression was similarly low in both wild-type and mutant leaf samples (Fig. 1-9). Transcript analysis revealed that the *OsBEIIa* mutation did not show typical RNA expression patterns in different samples, implying the possibility that other factors also determine the *sug-h* phenotype.

GUS was expressed under the control of the native *OsBEIIa* promoter in the wild-type background, and the results were consistent with the

qRT-PCR data. GUS expression was detected in leaf, stem base, node, and 20 DAF seeds (Fig. 1-8b–e). The expression levels of *OsBEIIa* were negligible in roots as determined by both qRT-PCR and GUS reporter analyses.

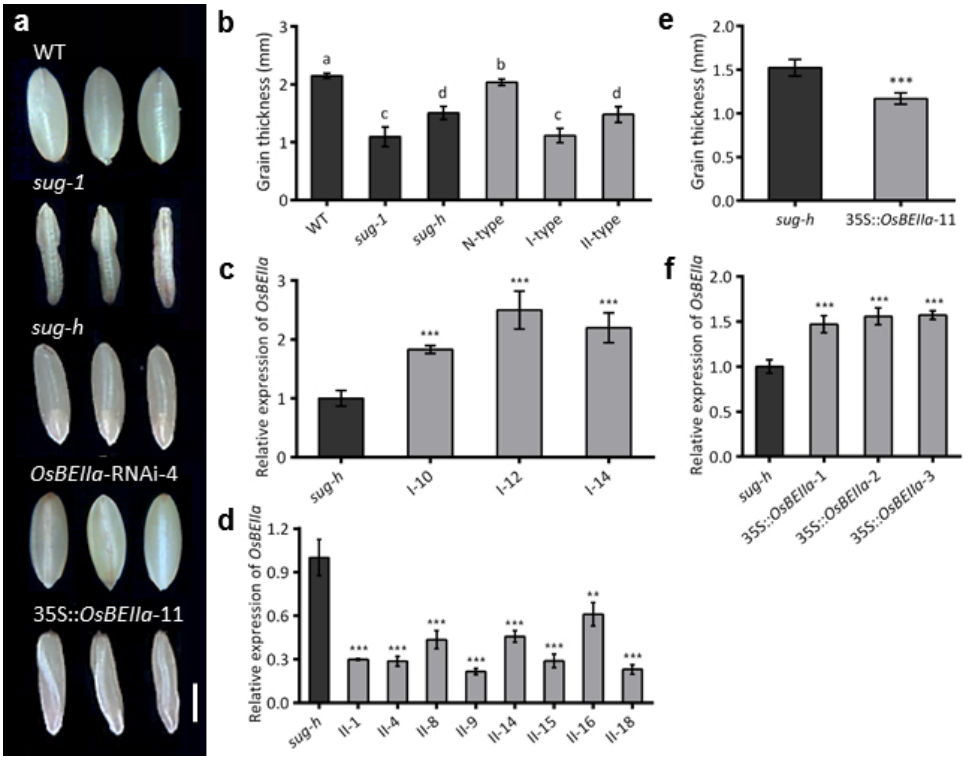

Figure. 1-7 Phenotype of transgenic seeds and complementation test of the *sug-h* mutation. **(a)** Grain morphologies of wild-type rice (Dongjin), *sug-1*, *sug-h*, and transgenic seeds. Bar=2 mm. **(b)** Comparison of grain thickness in F_2 seeds derived from *OsBEIIa*-RNAi-4/*sug-h*. Different letters denote significant differences. Replicate samples were measured 20 times. **(c)** Relative expression level of *OsBEIIa* in each F_2 plant derived from I-type seeds using qRT-PCR. The *sug-h* mutant was used as a control. Error bars represent SD for three technical experiments. Asterisks indicate statistical significance compared with the control, as determined by Student's *t*-test (***P<0.001). **(d)** Relative expression level of *OsBEIIa* in each F_2 plant derived from II-type seeds by qRT-PCR. Error bars represent SD for three technical experiments. Asterisks indicate statistical significance compared with the control, as determined by Student's *t*-test (**P<0.01, ***P<0.001). **(e)** Comparison of grain thickness in 35S::*OsBEIIa* T_1 and *sug-h* mutant seeds. Statistical significance was determined by Student's *t*-test (***P<0.001). Replicate samples were measured 20 times. **(f)** qRT-PCR analysis showed increased *OsBEIIa* expression in 35S::*OsBEIIa* T_1 plants. Each sample was pooled with three biological replicates; error bars represent SD for three technical experiments. Statistical significance was determined using Student's *t*-test (***P<0.001).

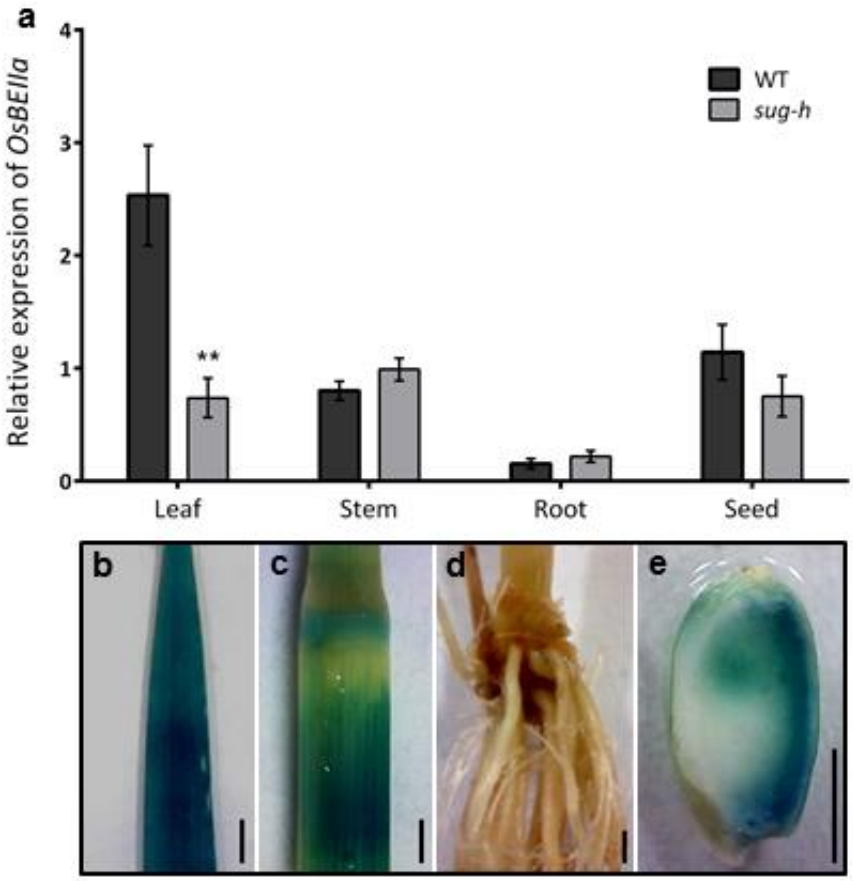

Figure. 1-8 Expression pattern of *OsBEIIa* gene **(a)** qRT-PCR analysis detected *OsBEIIa* transcripts in leaf, stem, root, and 10 DAF seeds. Data are mean ± SD (n=3). Statistical significance was determined using Student's t-test (**P<0.01). WT, wild-type rice (Hwacheong); DAF, days after flowering. **(b–e)** GUS expression was detected in leaf, root, stem base, and 20 DAF seeds in a transgenic plant expressing the *OsBEIIa* promoter::GUS reporter gene. Bars=2 mm.

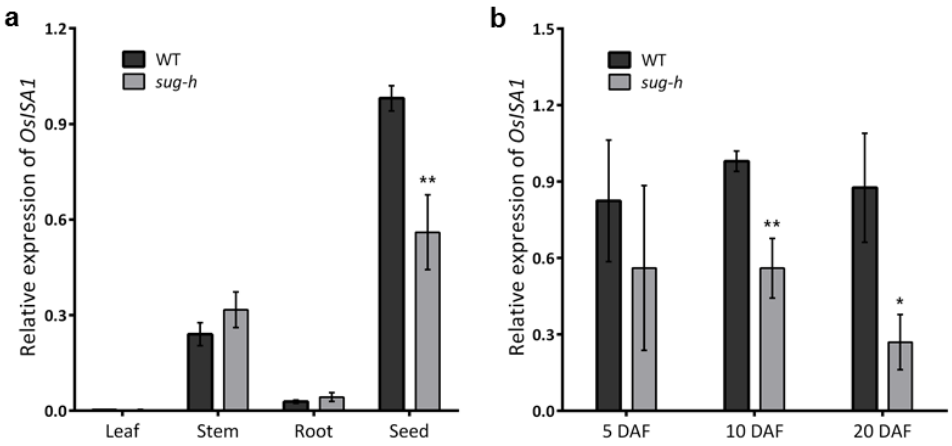

Figure. 1-9 *OsISA1* expression patterns in different organs and at different stages of seed development using qRT-PCR analysis. **(a)** Transcript levels decreased in seed (10 DAF) of the *sug-h* mutant. **(b)** *OsISA1* expression in 10 and 20 DAF seeds decreased in the *sug-h* mutant. All data are mean ± SD (n=3). Statistical significance was determined using Student's t-test (*P<0.05, **P<0.01). WT, wild-type rice (Hwacheong); DAF, days after flowering.

Debranching and branching enzyme activities in mutant endosperm

Native-PAGE/DBE and BE activity staining was performed to determine the change of enzyme activity in the *sug-h* mutant. Debranching enzymes, ISA and PUL, were detected as blue bands on the native gel containing potato tuber amylopectin stained with an iodine solution. ISA was visualized as three major bands with low mobility. ISA activity conspicuously decreased in both sugary mutants, as the same result with mapping on chromosome 8 (Fig. 1-10a).

Branching enzymes, BEI and BEII, were also detected on the native gel in distinct band patterns by iodine staining. While BEI activity was almost

same in both wild-type and *sug-1* mutant, BEII activity dramatically decreased only in the *sug-h* mutant (Fig. 1-10b). Unfortunately, we could not find the reason why two BEII isoforms, divided into BEIIa and BEIIb, were not seen on the gel. At any rate, it is obvious that BEII activity diminished in the *sug-h* mutant. Zymogram results imply that the difference between *sug-1* and *sug-h* mutant was caused by branching enzyme activity.

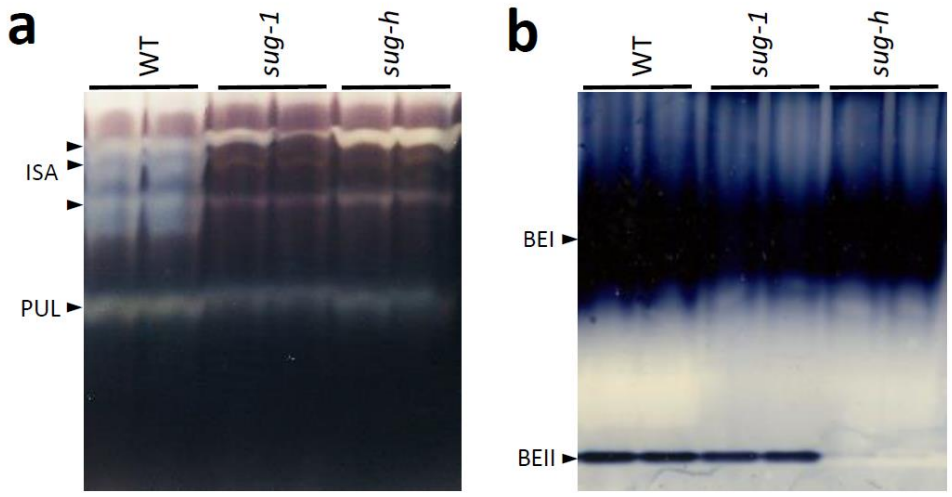

Figure. 1-10 Native-PAGE/activity staining of developing endosperm in wild-type and mutant seeds. **(a)** Native-PAGE/debranching enzyme (DBE) activity staining of rice endosperm at late-milky stage. The ISA and PUL activity bands are indicated by black arrows. **(b)** Native-PAGE/branching enzyme (BE) activity staining. The BEI and BEII activity bands are indicated by black arrows.

DISCUSSION

Genetic mapping of the *sug-h* rice mutant was used to identify and isolate two recessive genes, *OsISA1* and *OsBEIIa*. The *sug-h* mutant has a mild sugary phenotype, which preferentially accumulates desirable water-soluble carbohydrates rather than starch, and is more commercially viable than the *sug-1* mutant because it does not display excessive wrinkling, which interferes with milling. Segregation ratios of the F_2 population showed that the *sug-h* phenotype was controlled by complementary interactions between *OsISA1* and *OsBEIIa*. We demonstrated that *OsISA1* and *OsBEIIa* were associated with the genetic modifications that were responsible for the sugary endosperm phenotype. Although a single mutation in *OsBEIIa* did not affect endosperm phenotype, the mutation in *OsBEIIa* moderately recovered the sugary endosperm from the severe wrinkling caused by *osisa1*. Therefore, *sug-h* mutant seed maintains a sugary phenotype, but the seed quality is superior (less wrinkled) than that of *sug-1*.

There have been several reported mutants and transgenic rice related to sugary-type endosperm. Among them, severe sugary-type endosperm mutants, such as EM-914 (Nakamura et al. 1997; Wong et al. 2003), #1-1 (Tanaka et al. 2004), and *OsISA1* suppression and *OsISA2* over-expression transgenic lines (Utsumi et al. 2011), were similar to the *sug-1* mutant used in this study. Of them, phenotype of EM914 was governed by mutated *ISA1* while #1-1 and *OsISA2* over-expression line had different genes than *sug-1*. It is interesting that a mild sugary mutant, which was reported as a variation of *su-1* mutant by Nakamura et al. (1997), had the similar

phenotype to *sug-h* mutant although only *sug-1* locus was involved in the *su-1* mutant. The reason for the phenotypic similarity between *su-1* mutant by Nakamura et al. (1997) and *sug-h* mutant in this study remains to be comparatively studied. Recently, a rice novel endosperm mutant, named as *sugary-2*, was reported (Nakagami et al. 2016), in which the results on the activity of BE in the *sugary-2* mutant was unlike the *sug-h* mutant, indicating that the *sugary-2* mutant was different from the *sug-h* mutant.

Nakamura (2002) reported that inhibition of BEIIa activity caused low levels of short amylopectin chains with degree of polymerization (DP) ≤10 in rice leaf sheath, in which BEIIb is not expressed; however, the BEIIa-deficient mutant does not exhibit a significant change in amylopectin chain length profile in rice endosperm. Therefore, *OsBEIIa* function did not appear to play a direct role in the *sug-h* mutant endosperm. Consequently, we hypothesized that the amino acid substitutions in *OsISA1* and *OsBEIIa* changed the protein complex or enzyme interaction involved in starch biosynthesis, and might be responsible for the *sug-h* phenotype affecting amylopectin structure. Future studies will perform enzymatic analyses to test this hypothesis.

Previous studies investigated possible interactions between ISA and other enzymes. The debranching enzyme PUL was related to the sugary phenotype (Fujita et al. 2009). Amylose content, seed morphology, and starch granules of *pul* mutant lines were essentially the same as those of wild-type plants. By contrast, double *pul* and *isa1* mutant lines contained higher levels of WSPs and had shorter amylopectin chains with DP ≤7 in the endosperm compared with the *sug-1* parents, indicating that PUL can

partly compensate for starch biosynthesis. The absence of ISA activity primarily affected the sugary endosperm phenotype regardless of the presence of PUL activity. This result was very similar using the *sug-h* mutant; however, no differences in *OsPUL* sequences were identified in the Hwacheong wild type and the *sug-h* mutant.

FLOURY ENDOSPERM6 (*FLO6*) encodes a CBM48 domain-containing protein (Peng et al. (2014). FLO6 may act as a starch-binding protein interacting with ISA1, and may be a bridge between ISA1 and starch during starch biosynthesis. ISA1 may have interacting factors that mediate starch binding, and interacting enzymes that have not yet been elucidated. Previous research evaluated protein-protein interactions of starch biosynthetic enzymes. Crofts et al. (2015) reviewed that co-immunoprecipitation analysis revealed the following associations in rice: BEIIa-BEIIb, BEIIa-BEI, BEIIa-Pho1, BEIIa-SSI, and BEIIa-SSIIIa. The BEIIa-SSI interaction was also identified in wheat and maize (Tetlow et al. 2008). These results suggested that some isozymes involved in starch biosynthesis in rice formed active protein complexes. These combined results suggest a possible mechanism of BEIIa function in rice endosperm.

Phenotypic variation is a critical consideration for phenotypic analysis of sugary endosperm in *sug-h* populations because of environmental effects. Satoh et al. (2008) evaluated the effect of growth temperature on the frequency of various grain phenotypes and the extent of starch accumulation in the wild type and mutant, and reported that starch accumulation in the *pho1* mutant endosperm was affected by temperature. Similarly, the seed phenotypes of *sug-1* and *sug-h* mutants differed slightly between plants grown in the field and those grown in the green

house (data not shown). To reduce this phenotypic variation, all seeds from a whole main panicle of F_2 and F_3 plants grown in the field were used for genetic mapping. The mutants and wild-type seeds were grown together under the same conditions and prepared for phenotypic analysis at the same time. Future studies should assess the effects of environmental factors, especially temperature, on phenotypic variation.

In this study, we propose that mutated *OsBEIIa* plays a role in restoring the severely wrinkled sugary phenotype caused by *osisa1* in rice endosperm, although *OsBEIIa* mutation alone did not result in a significant phenotypic change. The observed complementary interaction between *OsISA1* and *OsBEIIa* provides novel insight into the roles of starch biosynthesis enzymes and their interactions. Our result can facilitate the breeding of functional rice cultivars with special nutritional qualities, and might be applicable to endosperm modification in other cereal crops.

CHAPTER II.

Genetic study on the Koshihikari-specific sequence related to eating quality in rice (*Oryza sativa* L.)

ABSTRACT

Rice eating quality is final goal in breeding of elite cultivar. Among premium rice cultivars, Koshihikari is a famous Japanease cultivar as superior quality rice. We focused on Koshihikari-specific marker, named 'P5', which was from the studies on development of markers for evaluation of eating quality, to identify the sequence related to good eating quality. P5 marker could not be found in Nipponbare sequence, thus, linkage mapping for investigation of P5 marker position was performed using two popolations with 72 STS markers. Two CSSL populations were also used to confirm the location of P5 marker position, thus, all data showed that P5 marker was on the long arm of chromosome 11. Whole genome sequencing and *De novo* assembly of Koshihikari was also carried out to idenetify Koshihikari-specific full sequence containing P5 marker region. As a result, this specific seqence was about 70 kb and replaced Koshihikari-specific sequence with Nipponbare. Semi-qRT-PCR results showed that only one gene was expressed among 10 predicted genes in this sequence. Effect of this sequence on rice eating quality was confirmed using NIL populations. Palatability of backcross lines substitued Koshihikari-specific sequence for Samnam (Korean *japonica* cultivar with poor eating quality) was

increased than that of Samnam, indicating that P5 sequence contributed the improvement of rice eating quality. These results will be helpful for elucidating a novel genetic factor for improvement of rice eating quailty by dissection on Koshihikari-specific sequence.

Key words: Koshihikari, eating quality, palatability, rice

INTRODUCTION

Rice is major crop in Asia for staple food, which has two cultivated species and 22 wild species and possesses big diversity for grain quality traits. As time goes by, eating quality of rice is increasingly important to meet the market demand. Data of market survey suggested that development varieties with improved cooking and eating quality have high economic values (Son et al. 2014). Therefore, one of the major goals in a breeding become to develop rice varieties of better eating quality to satisfy the requirements of both the food industry and consumers. Even though *indica* rice varieties are popular in many countries, consumers in Asian countries such as Korea, Japan, northern China, and Taiwan like *japonica* rice. Nowadays, more and more people who eat *indica* rice become prefer *japonica* rice mainly due to its moderate elasticity and stickiness.

Improvement of eating quality was considerably complicate because of many factors associated with cooking and eating quality. Eating quality is associated with such characteristics as glossiness, flavor, and stickiness. These characteristics reflect the chemical reaction that occurs during cooking of the rice grain, including hydration, gelatinization, length of cooking time, kernel elongation, and volume expansion (Juliano and Perez 1984). The physicochemical properties of rice starch in endosperm have been used as evaluation factors of eating quailty. Because the rice grain is mainly composed of starch, amylose content (AC), gel consistency (GC), and gelatinization temperature (GT) are the three major characters in eating quality (Bao et al. 2006b; Juliano 1985). In addition, pasting properties (PP) and protein content (PC) is regarded as eating quailty

affecting factors (Bao et al. 2006a; Ramesh et al. 2000).

There are several evaluation methods of eating quality, depending on various properties of rice. Sensory test is the simple and basic method by well-trained panels. This method has not yet been optimized as a routine tool because of its current limitations as being tedious, highly subjective, and time-consuming. Based on hydration retention on the cooked rice grains detected at a given electromagnetic wavelength, the palatability score measured by the Toyo taste meter is positively correlated with eating quality and used to evaluate rice grain quality. Japanese researchers have also reported that palatability values from the Toyo meter and palatability scores from the sensory test showed high positive correlation (Azuma et al. 1994; Tanaka et al. 1992). This indicates that the palatability value according to the Toyo meter can be used as a good measure of the eating quality of rice.

To complement the physicochemical analyses and sensory tests available to evaluate eating quality, DNA marker-based approaches have been developed (Ohstubo et al. 2003; Ohtsubo and Nakamura 2007). These methods offer the additional advantages of screening at early breeding generations as well as simplicity and accuracy. Several functional markers have also been developed to distinguish the physicochemical properties of rice, especially the effect of the waxy locus on PP (Larkin et al. 2003), that of SBE on starch viscosity (Han et al. 2004), and those of AC (Bao et al. 2006a) and starch synthase IIa (SSIIa) on GT (Bao et al. 2006b). Additional gene-tagged markers have also been developed from starchsynthesizing genes (Bao et al. 2006b; Han et al. 2004; Larkin et al. 2003). Lestari et al. (2009) reported that development DNA markers associated with eating

quality and to formulate a marker-based evaluation and prediction method of eating quality of cooked rice in *japonica* varieties. We focused on 'P5 marker' from Ohstubo et al. (2003) and Ohtsubo et al. (2002) in this study. Regression value of P5 marker in palatability by Toyo meter was 0.037, showing the largest portion in model equations for evaluating rice eating quality. However, P5 marker was in Koshihikari-derived cultivars only. This study aimed that identification of Koshihikari-specific sequence containing P5 marker and demonstration of the effects of this sequence associated with rice eating quality.

MATERIALS AND METHODS

Plant Materials

Two BC_1F_4 populations, 72 lines of Kohihikari/IR64//Koshihikari (KIRK) and 79 lines of IR64/Kohihikari//IR64 (IRKIR), were used for linkage mapping. Four CSSL lines from Rice genomic resource center (RGRC, Japan) were also used for screening of P5 marker (the Rice Genome Project of the National Institute of Agrobiological Sciences as the developer and the Rice Genome Resource Center as the provider of the material). The CSSLs contained 41 chromosomal segments of Nipponbare on a Koshihikari background (NKK), 48 chromosomal segments of Koshihikari on a Nipponbare background (NKN), 42 chromosomal segments of Koshihikari on a IR64 (KIK), 40 chromosomal segments of IR64 on a Koshihikari background (KII). We developed two BC_3F_3 and five BC_2F_3 lines of Samnam//Sanman/Koshihikari which have P5 region in Samnam (Korean *japonica* cultivar with poor eating quality) background. The lines were obtained through the backcross with RILs of Samnam/Koshihikari to Samnam, and marker assisted selection (MAS) with P5 marker. All populations were cultivated using conventional methods at the Experimental Farm of Seoul National University.

DNA extraction and PCR amplification

Total genomic DNA of all samples for genotyping were extracted from young leaves according to the modified method of McCouch *et al.* (1988). Polymerase chain reactions (PCR) were performed in a reaction volume of 20 µl containing 100 ng of template DNA, 0.1 µM each primer, 2.5 mM

dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 0.5 U Taq DNA polymerase. PCR amplification was carried out in a DNA Engine Tetrad 2 and Dyad Thermal Cycler (Bio-Rad, USA) using the following reaction conditions: 5 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 30 s at 58 °C, and 40 s at 72 °C; and 10 min at 72 °C for final extension. PCR products were separated on 2.5% (w/v) agarose gels containing 0.15 μg ml^{-1} ethidium bromide (EtBr) in 0.5 × TBE buffer.

Linkage mapping

A total 151 lines of a set of BC_1F_4 reciprocal population with Koshihikari and IR64 were genotyped by P5 marker and 72 subspecies-specific STS markers that were evenly distributed on the chromosomes (Chin et al. 2007). A linkage map included P5 marker was constructed by QTL IciMapping ver. 4.1 software (Meng et al. 2015).

Next-generation sequencing

Whole-genome shotgun sequencing of the *Oryza sativa* L. (spp. *japonica*) cv. Koshihikari was performed using the MiSeq platform.

Quality trimming of raw data

Preprocessing of raw data was performed using Trimmomatic V0.33 program (Bolger et al. 2014) for improvement of analysis accuracy. Options were applied as minimum quality of base[3], sliding window[4], average quality[20] and minimum read size[50].

De novo genome assembly of Koshihikari

The contigs were assembled using Newbler V2.9 (Roche, Germany), then

assembled into scaffolds using SSPACE V3.0 (Boetzer et al. 2011) program.

Chromosome walking and long-range PCR

Gap filling and scaffolding produced by *de novo* assembly was needed for identification of unknown sequence including P5 marker. Genomic DNA walking was performed using DNA Walking SpeedUp^{TM} Premix Kit (Seegene, Korea), following manufacturer's instruction. Large gap filling and scaffold ordering was carried out by conventional long-range PCR with PrimeSTAR GXL DNA Polymerase (Takara, Japan).

Gene prediction and semi-quantitative RT-PCR

Genes were predicted in the Koshihikari-specific sequence using Fgenesh software (http://linux1.softberry.com/berry.phtml). To demonstrate predicted transcripts, total RNA was extracted from Koshihikari and Nipponbare leaf samples at vegetative stage using MG Total RNA Extraction kit (Doctor Protein, Korea). The RNAs were converted into first-strand cDNA using M-MLV Reverse Transcriptase (Promega, USA). Semi-quantitative RT-PCR was performed in a reaction volume of 50 µl with *Ex taq* DNA polymerase kit (Takara, Japan) containing 10 × PCR buffer, 2.5 mM dNTP, and 0.5 U *Ex taq* polymerase. PCR amplification was carried out in a DNA Engine Tetrad 2 and Dyad Thermal Cycler (Bio-Rad, USA) using the following reaction conditions: 5 min at 94 °C; followed by 25 cycles of 1 min at 94 °C, 30 s at 56~60 °C, and 40 s at 72 °C; and 10 min at 72 °C for final extension. PCR products were separated on 2 % (w/v) agarose gels containing 0.15 µg ml^{-1} ethidium bromide (EtBr) in 0.5 × TBE buffer.

Non-coding RNA prediction and GO classification

Non-coding RNAs, containing miRNA, snRNA, snoRNA, tRNA, ta-siRNA and lncRNA were predicted using public database (Yi et al. 2015). Prediction was performed based on a default parameter, then data was arranged by number of mismatch. GO classification of miRNA target gene was also carried out using public database (http://ricedb.plantenergy.uwa.edu.au/).

Palatability evaluation using Toyo taste meter

Each samples were dehulled and polished in average 15% moisture content of grains. Palatability (glossiness) of cooked rice was measured in three replications using a Toyo taste meter (MA-90; Toyo, Japan), according to the operation manual.

RESULTS

Chromosomal position of Koshihikari-specific region

The P5 marker is Koshihikari-specific sequence, thus, could not be found in other cultivars included a Nipponbare cultivar. First, we performed constructing linkage map to investigate chromosomal position of P5 sequence using total 151 lines of two BC_1F_4 populations (KIRK and IRKIR, described in Materials and methods). Depending on linkage data between P5 and STS markers on each chromosome, chromosome 11 became the candidate location where P5 marker was (Fig. 2-1a). Second, screening of P5 marker in four CSSLs (NKK, NKN, KIK, and KII, described in Materials and methods) was conducted to confirm the position that was investigated in previous linkage map (Fig. 2-1b). The screening data also showed the same result with construction of linkage map. The results from linkage analysis indicated that P5 marker sequence was on the long arm of chromosome 11, specifically existed in Koshihikari chromosome.

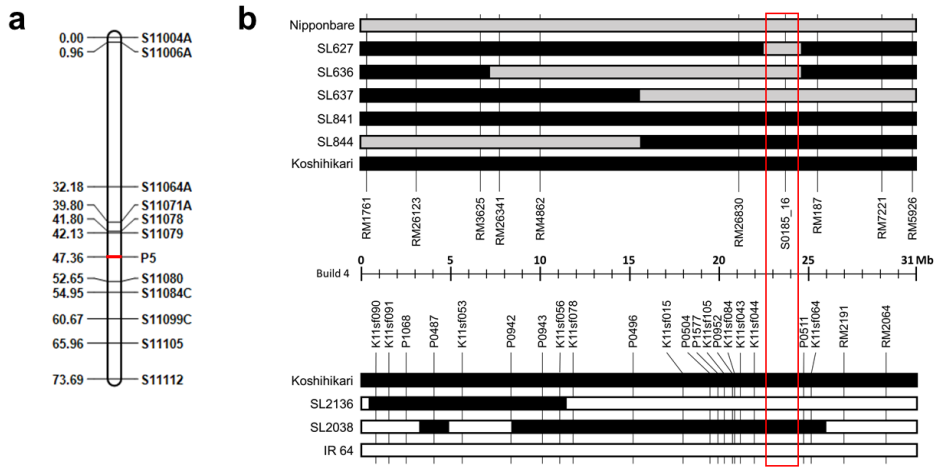

Figure. 2-1 Location of P5 marker in chromosome 11. **(a)** Construction of linkage map with P5 and STS markers using total 151 lines of two BC_1F_4 populations (KIRK and IRKIR) **(b)** Screening of P5 marker in four CSSLs (NKK, NKN, KIK, and KII). SL627, 636 and 637; SL841 and 844; SL2136; SL2038 were NKK, NKN, KII and KIK population, respectively. Red line and box indicate P5 position.

Identification of Koshihikari-specific sequence

To detect of the unknown Koshihikari-specific sequence containing P5, next-generation sequencing technology was adopted. For estimation of genome size, kmer size was 19, peak depth was 64, coverage was 78 ×, and estimated genome size was 407,741,111 bp. Paird-end and mate-pair information and assembly data were represented in Table 2-1 and Table 2-2. After *de novo* assembly, PCR product sequence of P5 marker were blasted against all scaffolds to detect large segment including P5 sequence. Three scaffolds which had about 32, 12 and 17 kb length were extracted,

then ordered, filled the gap and removed duplicates to complete full sequence by chromosome walking and long-range PCR technology. As a results, ~38 kb segment was obtained as Koshihikari-specific sequence (Fig. 2-2). The end of this segment was joined with Nipponbare sequence in 20.5 Mb region (based on IRGSP-1.0), however, the opposite side was still unclear the position and more extended sequence.

Sequence comparison between Koshihikari and Nipponbare was conducted by PCR with several pairs of primers designed in Nipponbare sequence from 20.35 to 20.55 Mb region on chromosome 11. According to presence and absence of PCR products, about 73 kb of Nipponbare sequence, from 20,433 kb to 20,507 kb, was replaced with unknown sequence of Koshihikari, including ~38 kb segment that we identified in this study. Sequence analysis indicated that Koshihikari had the structural variation in a long arm on chromosome 11, compared to Nipponbare, and ~70 kb region of Nipponbare was replaced with Koshihikari-specific sequence containing P5 marker in Koshihikari cultivar. Unknown sequence identification of ~35 kb sized gap in Koshihikari is still remains to be studied.

Table 2-1 Paired-end and mate-pair information for Koshihikari sequencing

	Number of reads	Total read length (bp)	Length coverage (X)
Paired-end	34,940,048	18,418,445,998	49.78
Mate-pair	66,555,826	6,749,975,818	314.79

Table 2-2 *De novo* assembly information of Koshihikari sequencing

	Contigs*	Scaffolds
Total number	34,373	7,184
Length (bp)	331,374,920	344,198,117
Largest length (bp)	103,121	974,491
N50 (bp)	16,485	137,807
Average Length (bp)	9,641	47,912

* Contigs and scaffolds over 1 k size were represented.

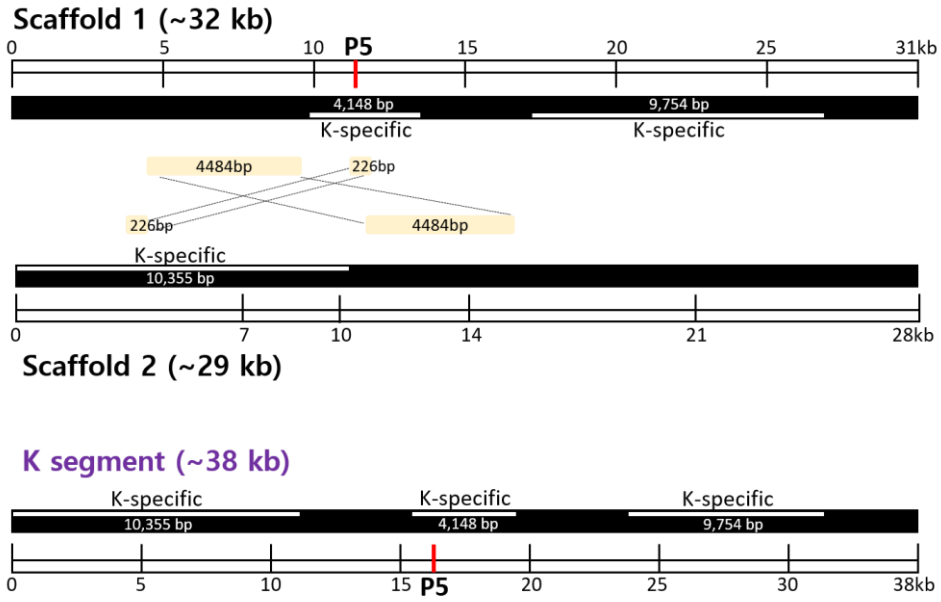

Figure. 2-2 Sequence extension and gap filling with scaffolds which were obtained by *de novo* assembly. Final size of Koshihikari-specific segment was about 38 kb.

Comparative study of Koshihikari-specific region

Comparison of genomic structure in other species was performed. First, there were duplicated segments from *O. sativa* (Nipponbare) sequence. These partial segments were blasted to chromosome 6, 11 and 12 with inconsistency. Second, partial sequence (~6 kb) was considerably matched with *O. glaberrima* sequence shared by Wang et al. (2014) (Fig. 2-3). PCR amplification of P5 product also succeeded with *O. glaberrima* DNA as well as *O. rufipogon* and *O. longistaminata* DNA (data not shown). These results implied that Koshihikari-specific sequence might be formed by partial duplication and fusion, and wild rice sequence, such as *O. glaberrima*, might give a clue for structural variation of Koshihikari-specific segment.

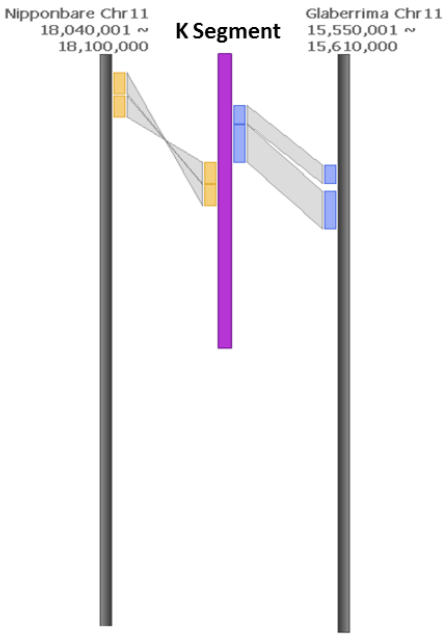

Figure. 2-3 Sequence comparison of Koshihikari-specific sequence (designated as K Segment in this figure) with *O. sativa* (Nipponbare) and *O. glaberrima*.

Gene prediction within Koshihikari-specific sequence

According to our hypothesis, we expected that structural variation of Koshihikari on chromosome 11 might have the relationship in good eating quality of Koshihikari. Therefore, gene prediction within unknown sequence was needed, as a result, eight genes in ~38 kb sequence were predicted and represented in Table 2-3 and Fig. 2-4. Transcripts were confirmed by semi-quantitative RT-PCR to check actual expression of each predicted gene. Among eight genes, only one transcript, plant transposon protein (K2 in the Table 2-3), was detected (Fig. 2-5). Although predicted genes in the Koshihikari-specific sequence seems not associated with eating quality, genes related to transposon in the P5 region might provide a clue for forming this cultivar specific sequence. The mechanism of Koshihikari-specific genes and segment effected on Koshihikari character is needed to be studied in the future.

Non-coding RNA prediction within Koshihikari-specific sequence

For detailed analysis within Koshihikari-specific sequence, non-coding RNA (ncRNA) prediction was performed using public database. Several ncRNA, containing 50 miRNAs, 6 snoRNAs, and 50 lncRNAs, were predicted based on genomic sequence blast. After filter within the default parameter, snRNA, tRNA, and ta-siRNA could not be found in Koshihikari-specific segment. The number of predicted ncRNAs depending on the mismatch was represent in Fig. 2-6.

The miRNAs usually have target genes which were regulated by the action of miRNAs. Among several target genes of each 50 predicted miRNA, total 67 genes which were known the function were filtered. These target

genes were classified into three classes, biological process, molecular function, and cellular component, on the basis of gene ontology (Fig. 2-7). Major functions of miRNA target genes were cellular process and catalytic activity, implying that these genes might be candidates related to eating quality.

Table 2-3 List of predicted genes in Koshihikari and Nipponbare within physical region from 22.26 kb to 22.35 kb of Nipponbare

Koshihikari

No.	Gene	Locus ID	Physical position (bp)	Direction	gDNA (bp)	cDNA (bp)	Amino acid(aa)	No. of exon
C1	Ankyrin domain containing protein	Os11g0549700	22268583-22274363	(+)	5,781	1,716	571	14
C2	Similar to tubulin alpha-6 chain(partial)	Os11g0549900	22275905-22277199	(+)	1,295	519	172	3
K1	NB-ARC domain			(-)	1,875	1,875	624	1
K2	Plant transposon protein			(+)	3,828	2,775	924	6
K3	RX-CC like, NB-ARC domain			(-)	861	861	286	1
K4	No conserved domain			(-)	237?	249	82	1
K5	Nicotianamine synthase protein			(-)	432	432	143	1
K6	No conserved domain			(+)	201	201	66	1
K7	Os11g0550800 partial match (50%)			(-)	387	387	128	1
K8	Os11g0550900 partial match (88%)			(-)	3,673	795	264	7
C3	No match	x	22350579-22351008	(+)	429	276	91	2
C4	RX-CC like superfamily	Os11g0551700	22356250-22356660	(+)	411	411	137	1

C1~C4; Common genes of both Koshihikari and Nipponbare in this region, K1~K8; Predictied genes in Koshihikari, N1~N10; Predictied genes in Nipponbare.

Nipponbare

No.	Gene	Locus ID	Physical position (bp)	Direction	gDNA (bp)	cDNA (bp)	Amino acid(aa)	No. of exon
C1	Ankyrin domain containing protein	Os11g0549700	22268583-22274363	(+)	5,781	1,716	571	14
C2	Similar to tubulin alpha-6 chain(partial)	Os11g0549900	22275905-22277199	(+)	1,295	519	172	3
N1	Similar to NB-ARC domain	Os11g0550100	22286977-22291701	(-)	5,536	2,208	735	3
N2	hypothetical protein	Os11g0550300	22295529-22297022	(+)	1,493	291	96	2
N3	Similar to LZ-NBS-LRR class RGA	Os11g0550500	22304189-22307756	(+)	3,568	3,012	1,003	4
N4	retrotransposon protein, putative, Ty1-copia subclass	x	x	(-)	3,589	3,252	1,083	4
N5	Similar to plastid-specific ribosomal protein 6	Os11g0550800	22318749-22321352	(-)	2,604	1,125	374	4
N6	Conserved hypothetical protein	Os11g0550900	22325618-22330265	(-)	4,648	852	283	9
N7	hypothetical protein	Os11g34960	22331779-22332545	(+)	767	174	57	3
N8	NB-ARC domain	Os11g0551300	22339035-22342952	(+)	3,918	2,748	915	4
N9	hypothetical protein	Os11g0551350	22343705-22345327	(-)	1,623	366	121	3
N10	hypothetical protein (InDel)	Os11g34990	22347872-22348253	(+)	382	261	86	2
C3	No match	x	22350579-22351008	(+)	429	276	91	2
C4	RX-CC like superfamily	Os11g0551700	22356250-22356660	(+)	411	411	137	1

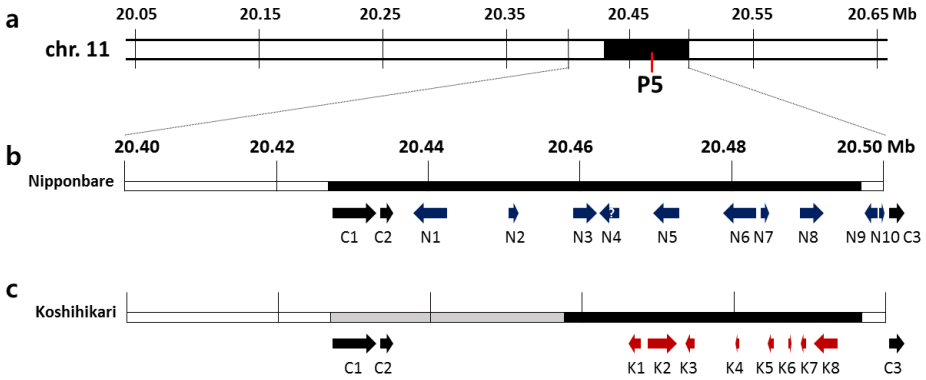

Figure. 2-4 Comparison of the long arm on chromosome 11 between Nipponbare and Koshihikari. **(a)** Location of P5 marker represented on the partial region on chromosome 11. **(b)** Genes are shown on Nipponbare chromosome. **(c)** Predicted genes are shown on Koshihikari chromosome. Black boxes are confirmed sequence and gray box is unknown sequence. C1~C3; Common genes of both Koshihikari and Nipponbare in this region, K1~K8; Predictied genes in Koshihikari, N1~N10; Predictied genes in Nipponbare.

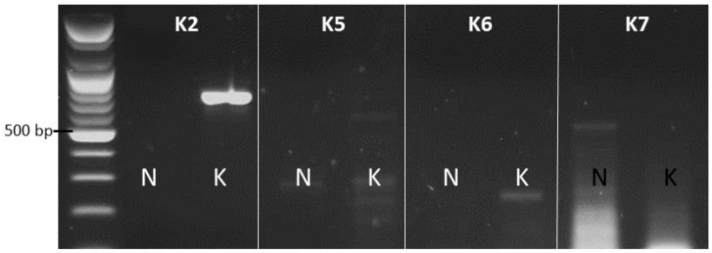

Figure. 2-5 Semi-quantitative RT-PCR for transcripts demonstration of each predicted gene in Koshihikari-specific sequence. Predicted genes, K2, K5, K6 and K7, are represented in Table 2-3. N; Nipponbare cDNA, K; Koshihikari cDNA sample.

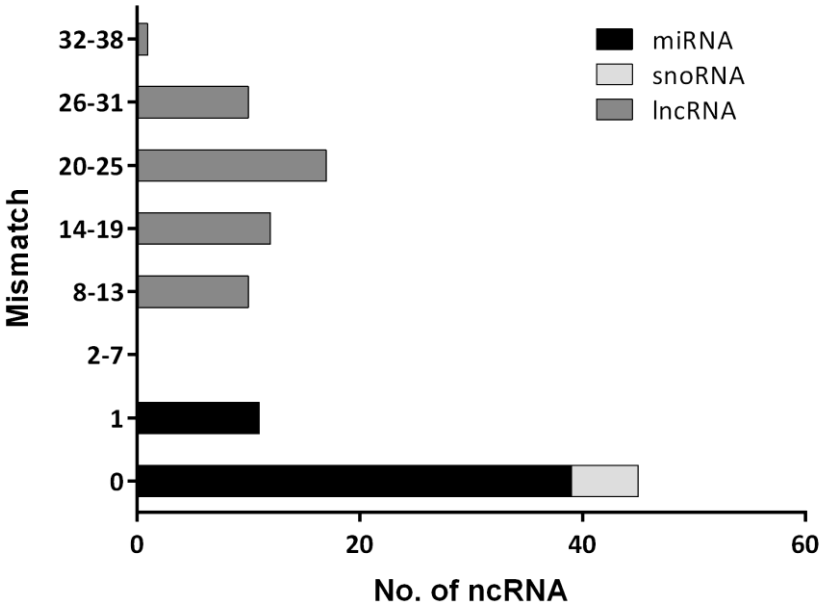

Figure. 2-6 The number of predicted ncRNAs depending on the mismatch within the Koshihikari-specific sequence.

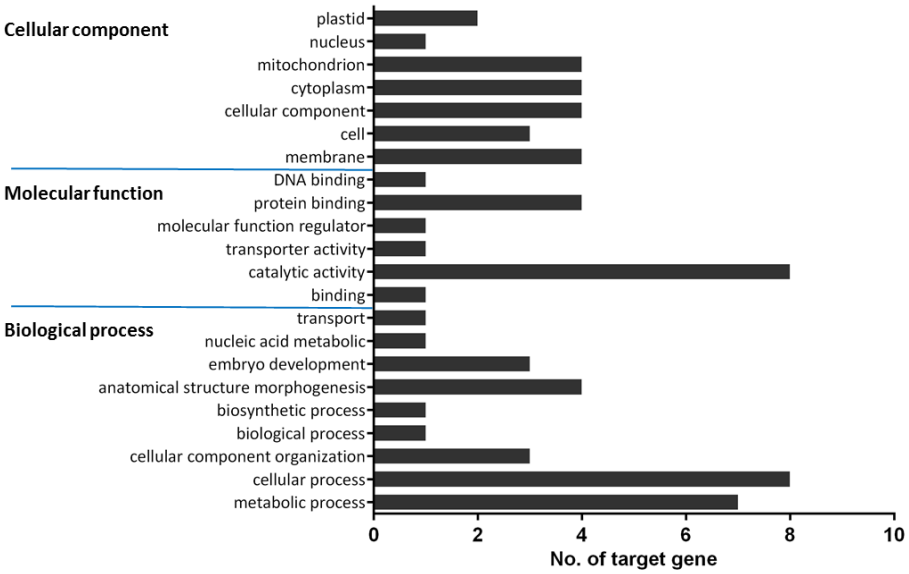

Figure. 2-7 GO classification for predicted target genes of each miRNA, depending on three classes.

Palatability evaluation of P5-substitution lines

To confirm of the effects of Koshihikari-specific sequence linked to P5 marker, two BC_3F_3 and five BC_2F_3 from Samnam//Samnam/Koshihikari were harvested and polished. Because correlated factor of P5 marker was the Toyo value in preceding research (Lestari et al. 2009), we evaluated palatability by Toyo taste meter. Among seven lines, palatability was significantly increased than Samnam in one BC_3F_3 and four BC_2F_3 lines (Fig. 2-8). Especially, Toyo value was recovered up to Koshihikari value in

960495 lines. Palatability evaluation results indicated that Koshihikari-specific P5 region had a positive effect to improve eating quality in rice.

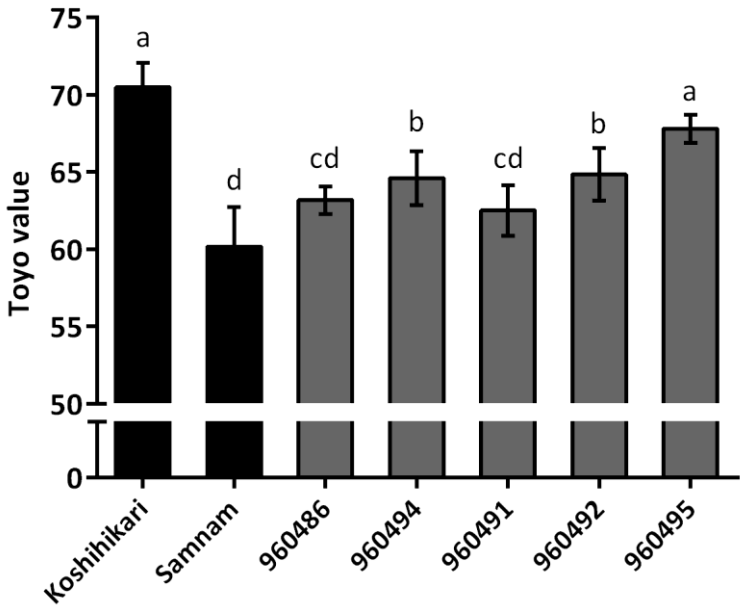

Figure. 2-8 Toyo value of five backcross lines containing P5 region in Samnam background and their parent.

DISCUSSION

Eating quality in rice is complex character which is difficult to directly estimate. Depending on the country, region, culture and individual preference, estimation of eating quality become varied. Because eating quality components are quite complicated, genetic factors which are considered important and fundamental for improvement eating quality are meaningful research concern. Although many QTLs and genes are isolated, researches on genetic factors related to eating quality still remained in genetic and genomic area. Characterization on eating quality is also difficult. Toyo taste meter value was usually used in evaluation of palatability. Several QTLs were detected by phenotyping with Toyo taste meter (Cho et al. 2014; Hsu et al. 2014; Kwon et al. 2011; Lee et al. 2003; Yun et al. 2016). Further study is needed to phenotype the components related to eating quality.

We analyzed the Koshihikari-specific sequence which expected to associate with rice eating quality. This sequence was from 557 bp, P5 marker PCR product, then extended up to ~38 kb nucleotide. We confirmed that this Koshihikari-specific sequence was related to good eating quality character through evaluation of palatability in BC_3F_3 and BC_2F_3 P5 introgression lines with Samnam background. According to previously reported resequencing data, there were cultivar-specific sequences or SNPs that were different with reference genome (Xu et al. 2011). As limited researches in cultivar-specific sequence, analysis of Koshihikari-specific sequence in this study will be expected to provide the clue for elucidating function of cultivar-specific sequences or SNPs. In addition,

structural variation in Koshihikari chromosome is also valuable to identify cultivar-specific function. The Koshihikari-specific sequence showed segment fusion form which was blasted with other chromosome sequences. We assumed that structural variation within the pedigree of Koshihikari was occurred, and the reasons of this variation were still remained to reveal. Identification for genetic action of Koshihikari-specific sequence is needed to prove of association with good eating quality in Koshihikari in the future.

It is interesting to trace where the Koshihikari-specific sequence derived from. Yamamoto et al. (2010) reported patterns of the pedigree haplotype blocks of Koshihikari and its related cultivars. We additionally confirmed that Koshihikari-specific sequence which was identify in this study was found in some wild rice, such as *O. rufipogon*, *O. longistaminata* and *O. glaberrima* (data not shown). Especially, it is known that *O. longistaminata* and *O. glaberrima* have evolutionally far distance with *O. sativa*. We could guess that this specific sequence was maintained during pedigree divergence into Koshihikari. Our results will helpful to support to rice evolutionary divergence by analysis of this sequence.

In this study, Koshihikari-specific sequence from eating quality-related marker, P5, was identified, and this sequence showed association with good eating quality. These results will be helpful for identifing novel genetic factors to improve rice eating quailty. Furthermore, marker development related to eating quality will be possible using genetic analysis data from this study.

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초록

벼 당질배유 유전자 *sug-h*와 식미연관 분자표지에 관한 유전적 연구

벼는 대표적인 식량작물로써 한국, 일본, 동남아시아 국가들을 비롯한 많은 나라에서 주식으로 이용되고 있다. 벼 육종은 시대가 바뀜에 따라 그 목표가 변해왔는데, 최근에는 고식미 및 고품질의 벼를 육종하려는 목표가 두드러지는 추세이다. 벼의 식미를 정의하기는 매우 어렵기 때문에, 본 연구에서는 식미에 관여하는 유전적 요인에 집중하여 연구를 진행하였다. 그 중, 벼 품질과 관련된 가장 직접적인 특성 중 하나는 배유 전분이다. 벼는 주로 종자, 특히 배유를 이용하는 작물이기 때문에 배유 전분의 특성 및 전분 생합성은 벼 품질에서 빠질 수 없는 부분이다. 본 연구에서는, 벼의 당질배유 성질에 관여하는 유전자를 동정한 내용과 고품질 식미에 관여할 것으로 보이는 고시히카리 품종 특이적 염기서열을 분석한 내용에 대해 논의하고자 한다.

벼 당질배유 돌연변이체는 정상 전분 대신 수용성 물질인 피토글리코겐이 배유에 축적되어, 주름지고 매우 납작한 종자 표현형을 보인다. 기존 연구에서 잘 알려진 당질배유 돌연변이체 *sug-1* (*sugary-1*)은 소화가 잘 되고 특정 영양학적 이점이 있음에도, 이러한 표현형 때문에 도정 등의 가공이 매우 어려워 상업적으로 이용할 수 없다. 이에 따라 본 연구실에서는 화학적으로 유기된 당질배유 돌연변이체를 분리하였다. 그 결과 기존 당질배유 돌연변이체인 *sug-1*과는 확연히 구별되는, 덜 주름지고 덜 납작한

표현형을 가져 상업적으로 유용한 *sug-h* (*sugary-h*)를 획득했고, 이를 재료로 하여 당질배유에 관여하는 유전자를 동정하게 되었다. 분리비 분석을 통해 *sug-h* 표현형은 두 개의 유전자가 상호작용을 통해 결정되는 것으로 예측되었고, 두 번에 걸친 유전자 지도에 기초한 유전자 분리를 통해 두 개의 유전자는 *Isoamylase1* (*OsISA1*)과 *Starch branching enzyme IIa* (*OsBEIIa*)임을 밝혔다. 형질전환 기법을 이용하여 유전자 기능을 확인해 본 결과, *OsISA1*에 의해 당질배유 특성이 결정되고, *OsBEIIa*에 의해 그 정도가 조절되어 보다 상업적으로 유리한 *sug-h*의 표현형이 결정된다는 것을 확인하였다. 본 연구는 벼 배유 전분 합성 경로에 새로운 시각을 제공해 줄 수 있을 뿐 아니라 기능성 쌀 개발에 좋은 재료가 될 것이라 본다.

벼 고식미 특성에 관한 유전적 연구를 위하여, 식미마커 개발에 관한 선행연구에서 고식미 특성에 영향을 미칠 것이라 통계적으로 분석된 분자마커인 'P5'로부터 이 연구를 시작하였다. 특히 이 마커는 고식미 품종으로 유명한 '고시히카리' 유래 품종에 특이적으로 존재하는 서열로, 기존 데이터베이스에서는 이 서열의 존재를 확인할 수 없다. 따라서 고식미 특성에 연관이 있을 것이라 예측되는 이 품종 특이적 마커 및 마커를 포함한 서열의 특징과 기능을 밝히는 것이 이 연구의 목적이다. 집단을 이용한 연관지도 작성 및 CSSL 집단 분석을 통해 이 특이적 서열은 벼 염색체 11번에 존재하는 것을 확인했고, 유전체 서열분석 및 *de novo* assembly를 이용하여 고시히카리 유래 품종에만 존재하는 특이적 서열 약 38 kb를 확인하였다. 이 서열에 대해 보다 구체적으로 파악하기 위하여 야생벼와의 서열 비교 및 유전자, non-coding RNA 예측 등을 수행하였으며 이는 추후에 실험적으로 증명해야 할 부분이다. 고시히카리 특이적 서열의 기능을 입증하기 위하여 식미가 좋지 않은 '삼남'에 고시히카리 특이적 서열이 삽입된 BC_2F_3 및 BC_3F_3 계통의 Toyo taste value를 측정하였다. 그 결과 이 서열이 삽입된 계통의 식미치가 삼남에 비해 증가한 것을 알 수 있었다. 본

연구는 벼의 고식미 특성에 관여하는 새로운 유전적 요인을 제안하고, 고품질 벼의 유전적 특징을 밝히는데 도움을 줄 수 있을 것이라 기대한다.

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