Production of High Amylose and Resistant Starch Rice Through Targeted Mutagenesis of Starch Branching Enzyme Iib by Crispr/cas9

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Abstract

Rice is the staple food for half of the world's population. Starch accounts for 80-90% of the total mass of rice seeds, and rice starch is low in resistant starch (RS) with a high glycemic index (GI). RS has gained important since it is beneficial in preventing various diseases. Starch branching enzyme IIb (SBEIIb) plays a key role in the amylopectin synthesis pathway in the endosperm of cereals. Down-regulation of *SBEIIb* in several important crops has led to high amylose, high RS and low GI starch.

In this study, we mutated *OsSBEIIb* in the *japonica* rice cultivar TNG82 through CRISPR/Cas9 and investigated the molecular and physicochemical modifications in *OsSBEIIb* mutant lines, e.g., gene expression, enzyme activity, apparent amylose content (AAC), RS and GI. As expected, the levels of modification in these starch related traits in heterozygous mutant lines were about half as those of homozygous mutant lines. Gene expression and enzyme activity of OsSBEIIb were down-regulated significantly while AAC and RS contents increased progressively from 17.4% and 0.5% in WT, respectively, to as high as 25.0% and 7.5% in heterozygous mutant lines and 36.0% and 12.0% in homozygous mutant lines. Consequently, with increased RS and decreased rate of reducing sugar production, GI progressively decreased in heterozygous and homozygous mutant rice endosperms by 11% and 28%, respectively. Our results demonstrate that it has huge potential for precise and efficient generation of high RS and low GI rice through CRISPR/Cas9 to provide a more suitable source of starch for type II diabetes.

Introduction

Diabetes mellitus is a chronic disease and type 2 diabetes (T2DM) is the most common form, representing 90–95% of diagnosed diabetes cases (IDF DIABETES ATLAS Ninth edition 2019). Glycemic index (GI) is a measure of postprandial blood glucose response after consumption of foods containing carbohydrate. GI is affected by starch chemical structure (e.g., amylose and amylopectin), and the level of GI affects the health of the human body. Foods with low GI can be absorbed slowly and help maintain blood sugar steady state, preventing various chronic diseases (Bjorck et al., 1994). Low GI foods have consistently shown beneficial effects on glycemic control in both the short term and the long term, also improve blood lipid concentration and prevent further diabetic complications. Thus, GI is a useful reference for individuals with T2DM to use as a dietary guideline (Riccardi et al., 2008).

Starch is the main carbohydrate in human diet and the main source of energy. Starch can be divided into amylose and amylopectin according to its structure. The difference in structure affects the rate of starch being digested. Starch with high amylose content (AC) and resistant starch (RS) has a lower GI value. RS refers to starch that cannot be digested and absorbed in the human small intestine (Englyst et al., 1992). RS functions similarly to soluble fiber; it helps feed the probiotic bacteria in gut and increases the production of short-chain fatty acids such as butyrate (Upadhyaya et al., 2016). Therefore, generating high amylose and RS content crops by traditional breeding or genetic technology is important to produce healthier foods and prevent diabetics.

Rice is a major staple food for half of the world's population, and rice starch accounts for 80–90% of the total mass of rice seeds (**Vandeputte** et al., **2004**). The physical and chemical properties of starch granules, such as AC, gelatinization temperature (GT), starch paste viscosity and amylopectin structure, affect the GI, eating and cooking quality of rice (Singh et al., 2006). Amylose is consisted of glucose residues linked by α (1→4) glycosidic bonds and form long linear chains with a degree of polymerization (DP) < 5,000. In contrast, amylopectin has amylose-like chains with additional branches formed by α (1→6) glycoside bonds with DP 5,000–50,000. Rice amylose is in the range of DP 230–10,000 and amylopectin chains are in the range of DP 6–120 (Fitzgerald et al., 2009). Cultivated rice in the world is mainly divided into *japonica* rice and *indica* rice. Starch from *indica* rice contains a higher amylose than that of *japonica* rice and has higher melting and pasting temperatures, gel hardness and lower pasting viscosity. It is well documented that amylose formation is controlled by one major locus *Wx* in the rice genome which encodes the granule-bound starch synthase I (GBSSI) protein. *Wx* has three major *Wx* alleles, *Wx*-I, *Wx*-II and *Wx*-III. *Wx*-I bears a loss-of-function mutation that results in glutinous rice varieties with extremely low AC (< 2%), *Wx*-II shows a leaky phenotype that leads to a medium level of AC (< 20%) which is widely found in *japonica*, and *Wx*-III functions as the WT allele with high AC (> 20%) mainly distributed in *indica* rice (Tian et al., 2009). Starch synthase IIa (SSIIa) and starch synthase IIIa (SSIIIa) are mainly present in endosperm. The main function of SSIIa is to use medium chains to synthesize long chains of amylopectin with DP of 16–21 and SSIIIa is mainly synthesize long chains of amylopectin with DP > 30. Down-regulation of *OsSSIIa* or *OsSSIIIa* significantly increases the expression level of *OsGBSSI*, apparent amylose content (AAC) and AC but decreases the amylopectin content in rice (Fujita et al., 2007). Starch branching enzyme (SBE) is the only enzyme capable of forming the branch linkages in amylopectin. The main function of SBE enzyme is to cut the α(1→4) glycosidic bonds and pass the broken short chain for reconnection to the acceptor chain through α(1→6) glycosidic bonds to form amylopectin (Tian et al., 2009). There are two classes of SBEs: SBEI and SBEII. In the endosperm of monocots, SBEII is divided in SBEIIa and SBEIIb (**Nakamura** et al., **2018**). In rice and maize endosperms, SBEIIb is the major isoform (Ohdan et al., 2005). The mount of SBEIIa is 2–3 fold higher than SBEIIb in wheat endosperm (Regina et al., 2015), while the activity of SBEIIa is equal to that of SBEIIb in barley endosperm (Regina et al., 2010).

Among most cereals, amylose is the main linear component of cereal starch and usually accounts for 15–25% (Ball et al., 1998). The AC of wild and cultivated rice ranges from 0–30%. Overexpression of the appropriate *Wx* gene which encodes the GBSSI can further increase the level of amylose (Hanashiro et al., 2008) while down-regulation of the expression of the enzymes involved in the biosynthesis of amylopectin like SBEI and SBEIIb thereby reduces the synthesis of amylopectin and increases the proportion of amylose and RS in cereals (Wei et al., 2010a; Butardo et al., 2011). Inhibiting the expression of *SBEIIb* genes affects the synthesis and structure of amylopectin. Study in wheat showed that inhibition of both *TaSBEIIa* and *TaSBEIIb* at the same times significantly increases long side-chain branching of amylopectin with DP > 24, AC and RS (Regina et al., 2015). Inhibiting *StSBEIIb* in potatoes also increases the average length of amylopectin branch chains in its starch, leading to an increase in AAC (Jobling et al., 1999). In addition, overexpressing *StSBEII* in potato leads to an increase in the number of amylopectin

chains of DP ≤ 12 and causes a particularly large increase in those of DP6 with significantly decreased AC (Brummell et al., 2015). The rice *OsSBEIIb* mutants through antisense RNA show significant change in the structure of amylopectin and content of short side chains with a significantly reduced DP of 8 ~ 12 in the endosperm (Zhu et al., 2012). Compared with WT, the endosperm of the transgenic rice with suppressed expression of *OsSBEIIb* gene has a higher AC, RS, gelatinization temperature, and stronger resistance to acid hydrolysis and enzymatic hydrolysis. Starch granules with a higher ratio of long chains of amylopectin are more resistant to gelatinization (Jane et al., 1999). Consistently, deletion or suppression of *OsSBEIIb* by chemical mutagenesis, RNAi and CRISPR/Cas9 targeted editing in rice causes a significant increase in the amylose and RS contents and a significant decrease in amylopectin content. Significant increase of AC also changes starch chemical properties, e.g. decreased swelling power and viscosity or increased gelatinization temperature (Wei et al., 2010a; Butardo et al., 2011; Yang et al., 2012; Sun et al., 2017; Baysal et al., 2020). These studies suggest that overexpression of GBSSI to increase amylose biosynthesis or down-reulation of *SBEIIb*, *SBEI*, *SSIIa* and *SSIIIa* to inhibit amylopectin biosynthesis can effectively increase AC in the endosperms of cereal crops.

Foods rich in RS can lead to lower blood sugar and insulin response, and reduce the risk of type II diabetes, obesity and cardiovascular disease (Rahman et al., 2007). A positive relationship between RS and AC has been established through several studies. Generating the high amylose crops through inhibition of the *SBEIIb* gene leads to increase RS with a strong anti-digestive properties, with beneficial to human health. A large number of high amylose crops have been generated through down-regulation of *SBEII* gene by chemical mutagenesis, RNAi and CRISPR/Cas9, including high amylose and RS wheat (Regina et al., 2015; Li et al., 2020), rice (Satoh et al., 2003; Shu et al., 2006; Butardo et al., 2011; Zhu et al., 2012; Wei et al., 2010a; Sun et al., 2017; Baysal et al., 2020), maize (Boyer and Preiss, 1978; Li et al., 2008; He et al., 2020), barley (Regina et al., 2010) and potato (Jobling et al., 1999; Tuncel et al., 2019). These crops can be the healthy source of carbohydrate food for patients with chronic diseases, such as diabetes and cardiac disease.

Traditional cross and mutation breeding are restricted by the large-scale screening, time-consuming, low efficiency and genetic barrier and cannot keep pace with the demand for increased crop production. On the other hand, genetic engineering provides big potential to increase crop productivity by overcoming these limitations (Mehta et al., 2020). In particular, genome editing techniques that can change specific positions in the genome have gained popularity in recent years for improving crop traits. CRISPR/Cas mediated gene editing is simple and easy to implement with a high efficiency, and therefore become a new tool leading the gene editing technology. CRISPR/Cas9 is suitable and efficient for crop breeding and exploring the functions of plant genes for crop improvement (Bortesi and Fischer, 2015). Thus, CRISPR/Cas9-mediated gene editing technology has the potential to greatly facilitate plant breeding. AC is controlled by a single dominant *Waxy* gene, which encodes a GBSS in rice. The loss of function caused by CRISPR/Cas9 of rice *wx* genes reduces the AC (Zhang et al., 2018). On the other hand, CRISPR/Cas9-mediated loss-of-function mutations of starch branching enzymes *SBEI* and *SBEIIb* in rice leads to higher AC and RS (Sun et al., 2017). Strong reduction in both *StSBE*1 and *StSBE2* in tetraploid potatoes through CRISPR/Cas9 also creates high amylose potato with recreation in short amylopectin chains, an increase

in long chains and a large reduction in branching frequency relative to WT starch (Tuncel et al., 2019). Targeted mutagenesis of *TaSBEIIa* by CRISPR/Cas9 in both winter and spring wheat varieties has generated transgene-free high-amylose wheat which also significantly increased RS, protein and soluble pentosane contents (Li et al., 2020).

In this study, we report the feasibility of creating high amylose, high RS and low GI rice plants through CRISPR/Cas9-mediated target gene editing of *OsSBEIIb* in the *japonica* rice cultivar TNG82. CRISPR/Cas9 has advantages over other means of gene editing, including low cost, easy operation, and high efficiency, etc. We also define the role of SBEIIb in determining the different molecular and physical modifications of *OsSBEIIb* gene between homozygous and heterozygous mutants, as shown in gene expression level, enzyme activity, amylose, RS, and physicochemical properties of starch. Thus, this work enables the provision of a more suitable source of starch for type II diabetes and advances the work of breeding in the future.

Materials And Methods

Vector construction

The sgRNA-Cas9 plant expression vector was kindly provided by Prof. Yaoguang Liu, South China Agriculture University and reconstructed by Academia Sinica for this work. The vector was reconstructed by inserting synthesized oligos into a *Bsa*I site of the vector *p*YLCRISPR/Cas9Pubi-H, which contains a codon-optimized Cas9 driven by a maize ubiquitin promoter, a sgRNA scaffold directed by a rice U6a promoter, a selectable marker gene (*hptII*) driven by a CaMV 35S promoter and the backbone of the binary vector *p*CAMBIA1300 (CAMBIA, Canberra, Australia) (Ma et al., 2015). The sgRNA and off target site analysis were designed by CRISPR-P 2.0 design tool (Liu et al., 2017). The 2 editing target sites were designed on exon 3 and intron 3 of *OsSBEIIb* gene.

Rice transformation

Rice transformation through *A. tumefaciens* was carried out according to previous methods (Ku et al., 1999; Yeh et al., 2015). Calli induced from immature seeds of the *japonica* cultivar rice TNG82 (*Oryza sativa* L.) were used for rice transformation. The compact embryogenic calli were co-cultured with *A. tumefaciens* strain AGL1 carrying the plasmid *p*YLCRISPR/Cas9Pubi-H and incubated at 28°C in dark for 3–4 days. After co-cultivation, calli were thoroughly washed with 250 mg L^{-1} cefotaxime in sterile distilled water and transferred to a selection medium containing 50 mg L^{-1} hygromycin B (Invitrogen®) and 250 mg L^{-1} cefotaxime at 28°C in light for one month. Healthy hygromycin-resistant calli were subsequently transferred to a 1/2 MS regeneration medium supplemented with 2.5 mg L^{-1} kinetin, 1 mg L^{-1} NAA and 50 mg L^{-1} hygromycin. Transgenic plantlets were transferred to a hormone-free 1/2 MS medium without hygromycin in magenta boxes for 10–14 days to promote root growth. Calli selection and plant regeneration were conducted in a growth chamber at 28°C and 60% relative humidity under a 16 h/8 h light/dark photoperiod. Transgenic seedlings were transplanted in soil, cultured in the

greenhouse or field between May and October. Self-pollinated seeds obtained from greenhouse-grown T0 plants were used for analysis of gene expression level and SBEIIb enzyme activity while the seeds obtained from field-grown plants were used for analysis of grain composition and molecular structure of starch.

DNA extraction, PCR, restriction enzyme digestion and screening of mutant plants by sequencing

To detect mutations in transgenic lines, genomic DNA was isolated from leaf tissue or calli of transgenic and WT (wild type) plants, as described (Sheu et al., 1996). Two hundred ng of genomic DNA was used as template for PCR amplification using Taq DNA Polymerase Master Mix RED (AMPLIQON®). Specific primers used in PCR analysis **(Supplemental Table 1)** included the primers gSBE3-F2/gSBE3-R2 for flanking the sequence of target site 1 (exon 3) and the primers gSBE3-F1/gSBE3-R1 for flanking the sequence of target site 1 and target site 2. The PCR products amplified by the primers gSBE3-F2/gSBE3-R2 using calli genomic DNA as template were used for restriction enzyme digestion analysis to confirm mutations. PCR was performed in a thermal cycler under the following conditions: 94°C/5 min, 30 cycles of 94°C/0.5 min, 55°C/0.5 min and 72°C/0.5 min, and a final extension of 72°C/5 min. The PCR products were digested with *Dde*I; and mutants displayed distinct band patterns on agarose gel, as compared to WT. Regenerated T0 plants from confirmed calli mutant lines were subjected to sequence analysis. The DNA fragment across the target site 1 and target site 2 was amplified by the primers gSBE3-F1/gSBE3-R1 using leaf genomic DNA in PCR reaction: 94°C/5 min, 30 cycles of 94°C/0.5 min, 60°C /0.5 min and 72°C/0.5 min, and a final extension of 72°C/5 min. PCR products were cloned into the TA cloning vector T&A™ (Yeastern Biotech Co., Ltd) for sequencing

Analysis of grain composition

Seeds were harvested and dried at 37°C for at least 3 days. Hundred-grain weights (g) of selected representative transgenic lines was measured in triplicate. The absolute digimatic caliper (Mitutoyo, Japan) was used to determine grain appearance and dimension. The opacity of seeds was investigated using the chalkiness index following the standard of Australian rice industry.

Analysis of OsSBEIIb **gene expression by qRT-PCR**

Total RNA was isolated from endosperm tissues, as described by Wang and Vodkin (1994). Total RNA was first treated with RNase-free DNase I to remove genomic DNA and the resulting RNA was used for first-strand cDNA synthesis with a random primer **(Supplemental Table 1)** and M-MLV reverse transcriptase (Promega) (Chen et al., 2006). Two µg cDNA was used as a template for gene expression assay by qRT-PCR; and the protocols of Livak and Schmittgen (2001) and Yeh et al., (2015) were followed using specific primers qcSBE3-F2/qcSBE3-R2 for *OsSBEIIb* and primers qcSBE1-F1/qcSBE1-R1 for *OsSBEI* **(Supplemental Table 1)**. Expression of *17S rRNA* (accession number X00755) was used as an internal control for normalization of expression levels.

Activity assay of starch branching enzyme (SBE)

The method for assaying the total activity of SBE in rice grains (Sun et al., 2011) includes the activities of SBE1, SBEIIa, and SBEIIb together.

Analysis of apparent amylose content (AAC)

AAC was determined by the standardized protocol ISO 6647-1 (Juliano et al., 1981).

Analysis of total starch, amylose content (AC), resistant starch (RS) and amylopectin content

The total starch, AC and RS contents in the rice flour were measured *in vitro* with the starch assay kits Megazyme K-STAR, K-AMYL and K-RSTAR (Megazyme, Wicklow, Ireland), respectively. The amylopectin content was indirectly determined by subtracting the amylose percentage from the total starch percentage of the sample.

Measurement of the rate of reducing sugar production

The *in vitro* digestibility of rice flour was determined as described previously (Butardo et al., 2011). The rate of reducing sugar production was calculated as follows: $(R_{180} - R_{10})/170$ (mg^{-1} min^{-1}); S_{180}, reducing sugar content at 180 min (mg); R_{10}, reducing sugar content at 10 min (mg).

Rapid visco analysis

The American Association of Cereal Chemists (AACC) 61 − 02 standard measurement method was slightly modified for viscosity analysis. Three g of rice flour was mixed with 25 mL pure water in a measuring cup for measurement of viscosity using RVA-4 (Newport Scientific, Australia) rapid viscosity analyzer. After incubating at a fixed temperature of 50℃ for 1 min, the temperature was increased to 95℃ at a rate of 12℃/min and maintained at 95℃ for 2.5 min. Then the temperature was reduced to 50℃ at 12℃/min and maintained at 50℃ constant temperature to the end of the measurement, the total measurement time of the sample was 12 min and 30 sec. The Thermocline for Windows version 2.4 software was used to record the time, temperature, viscosity, and other information from the RVA instrument in the entire measurement process. The built-in analysis tool of the software analyzed the following seven fast viscosity characteristic values: (1) Peak viscosity (PKV): the first high viscosity after the starch solution thickens, (2) Hot paste viscosity (HPV): the lowest point of viscosity after starch is completely gelatinized, (3) Cool paste viscosity (CPV): the high viscosity produced by the heated starch solution after cooling, also known as final viscosity, (4) Breakdown viscosity (BDV): PKV - HPV, and (5) Setback viscosity (SBV): CPV - PKV.

Measurement of glycemic index (GI) through static in vitro **digestion**

The consensus INFOGEST protocol was followed for the static *in vitro* digestion (Minekus et al., 2014). The obtained values of starch available were normalized to percentage of hydrolyzed starch.

Calculation of glycemic index (GI)

Glucose concentration quantified during the digestion process was normalized to percentage of total starch hydrolyzed, so that the GI index of the samples can be determined using the area under the curve (AUC) (Fernandes et al., 2020).

Data Analyses

Grain morphology, starch molecular and physiochemical properties of TNG82 (untransformed wild type), TNGS14 (*indica* rice variety) and *OsSBEIIb* mutant lines were analyzed by one-way variance analysis (ANOVA). Standard deviation was used to represent error values or error bars. Student's t-test was used to test significant difference in starch molecular and physiochemical properties between TNG82 and *OsSBEIIb* mutant lines.

Results

CRISPR/Cas9-mediated mutagenesis of OsSBEIIb in rice and production of transgenic plants

We targeted the third exon (Target 1) and third intron (Target 2) of rice *OsSBEIIb* gene (EnsemblPlants no Os02g0528200) to make long deletion mutations through CRISPR/Cas9 mediated editing (Fig. 1A). Target 1 sequence contains a restriction site for screening of mutations using PCR-based restriction enzyme (PCR/RE) digestion (Fig. 1A). The CRISPR/Cas9 construct for rice transformation, kindly provided by Academia Sinica, contains two gRNA cassettes in the vector *p*YLCRISPR/Cas9Pubi-H (Fig. 1B).

The CRISPR/Cas9 vector was transformed into embryogenic calli of the *japonica* rice cultivar (TNG82), through *Agrobacterium*-mediated method, and multiple transgenic lines were obtained after three rounds of selection on hygromycin-containing medium (Yeh et al., 2015). In total, 15 independent *OsSBEIIb* mutant rice lines were selected from 46 hygromycin-resistant transgenic calli through PCR/RE assay for detailed analysis. Our sequence analysis indicated that, of these 15 independent transgenic plants, 1 line (6.6%), 11 lines (73.3%), and 3 lines (20.0%) are genotypically homozygous, bi-allelic, and heterozygous mutants, respectively, at the first target site on exon 3 of *OsSBEIIb* (Fig. 1C, D).

We obtained long deletion mutants at a single or both alleles in which Cas9 cuts the two target sites at the same time, e.g., Line-34, 60 and 64 **(Table 1)**. However, some mutants harbored large insertions, e.g., Line-60 had 210 bps insertion at the first target site that contains a *Dde*I cutting site. In addition, there were several short insertion and deletion mutants with 1–26 bps. Line-64 is considered as heterozygous for phenotype because it has 221 bps deletion at one allele and 3 bps deletion at the other allele of Target 1 **(Table 1)**, which may not change OsSBEIIb function. Representative *OsSBEIIb* homozygous and heterozygous mutant lines were chosen for comparison with WT and *indica* cultivar TNGS14. At the first target we selected five homozygous (Line-33, 34, 42, 43, 60) and two heterozygous (Line-37, 64) *OsSBEIIb* mutant lines for phenotypic analyses, such as gene expression, grain morphology and starch physicochemical properties. CRISPR-Cas9-based genetic screens is a powerful new tool in biology, but the on-target activity and off-target effects of individual sgRNAs can vary widely. CRISPR-P2 tool was used to predict potential off-target site editing of the two target sites for *OsSBEIIb*, and site-specific genomic PCR

and DNA sequencing were used to investigate whether the predicted off-target sites were also edited. The results showed that no mutations are detected at the putative off-target loci in the genome of the seven *OsSBEIIb* mutant lines obtained in this study **(Supplemental Table 2)**.

OsSBEIIb **expression and SBE activity during rice grain filling in WT and seven** OsSBEIIb **mutant lines**

Quantitative real-time PCR (qRT-PCR) analysis showed that the expression levels of *OsSBEIIb* in both homozygous and heterozygous mutant lines were significantly lower than that of WT **(**Figure 2A**)**, with very low levels in the five homozygous mutant lines (Line-33, 34, 42, 43, 60) to about 2.5-fold lower in the two heterozygous mutant lines (Line-37, 64) in comparison to that in WT. As expected, the expression of *OsSBEIIb* in the homozygous mutants was almost completely knocked down since rice genome contains only one copy of the gene **(**Yang et al., 2012). The expression levels of *OsSBEI* were not altered in these mutant lines **(Supplemental Figure 1)**. The total enzyme activities of SBE in young rice grains 20 and 25 days after flowering were compared between WT and mutant lines **(**Figure 2B**)**. For both WT and the mutant lines, the total activities of SBE at 20 days were higher than those of SBE at 25 days after flowering. Both homozygous and heterozygous *OsSBEIIb* mutant lines showed significantly lower activities than that of WT **(**Figure 2A**)**. Compared to WT, the five homozygous and two heterozygous mutant lines exhibited nearly 3 and 2-fold decreases in total SBE activities, respectively. The decreases in total SBE activity among the mutants were mainly due to the lower *OsSBEIIb* expression levels with a positive correlation coefficient of r = +0.95 **(Supplemental Figure 2A)**. The substantial activities remaining in the mutant grains are presumably due to the normal expression of other SBEs **(**Baysal et al., 2020).

Grain morphology and starch physicochemical properties

WT, *indica* cultivar TNGS14, 2 heterozygous mutant lines (Line37, 64) and 2 homozygous mutant lines (Line-34, 60) that have long deletion at the first target site, and Line-42 that has 5 bps deletion at each alleles at the first target site were selected for comparison of their grain morphology and starch physiochemical properties. The grain weight, length, width and thickness were significantly lower in the homozygous mutant lines than those of WT, while there were no significant differences in these traits between the heterozygous mutant lines and WT. The grains of the heterozygous mutant lines were chalky, while the grains of the homozygous mutant lines were opaque throughout **(**Fig. 3**)**. In contrast, WT grains appeared uniformly translucent. The Onset gelatinization temperature of homozygous mutant lines was significantly higher than that of WT whereas, it was slightly higher in heterozygous mutant lines than WT **(Table 2)**. The predicted glycemic indexes (GI) of the homozygous (average 55.5) and heterozygous mutant lines (average 68.5) were significantly lower than that of WT (77); and the GI values of the two heterozygous mutant lines were similar to that of the *indica* cultivar TNGS14 **(Table 2)**. The decreases in GI among the mutants are mainly due to the increases in RS content with a negative correlation coefficient of r = -0.97 **(Supplemental Fig. 2K)**.

Apparent amylose content (AAC), amylose content (AC), total starch content and the ratio of amylopectin/amylose in indica **rice cultivar TNGS14, TNG82 (WT) and seven** OsSBEIIb **mutant lines**

The ACs of WT and the *indica* rice cultivar TNGS14 were 15.8% and 26.8%, respectively. As expected, *indica* rice tends to have a much higher AC than *japonica* rice. Both AAC and AC increased significantly in the seven mutated lines, with AAC ranging from 23.1 to 23.6% in the two heterozygous and from 34.0 to 36.0% in the five homozygous mutant lines (Fig. 4A**)**. On average, the AC increased from 15.8% in the WT to 24.1% (1.53 fold) in the heterozygous and 30.8% (1.95 fold) in the homozygous mutant lines (Fig. 4B). The increases in AC in the grains of these mutant lines did not affect the total starch contents; the total starch contents in the mutant lines were similar to that in WT (Fig. 4C). As expected, the ratio of amylose/amylopectin increased significantly in the mutant lines, relative to WT, ranging from 21.0 in TNG82 to 32.7–38.4 in the heterozygous mutant lines and 50.4–54.4 in the homozygous mutants (Fig. 4D). Taken together, these results clearly show that a total knockout of *OsSBEIIb* in rice can increase AC from 15.8 to 31–35% in TNG82 which are mainly due to the decreases in *OsSBEIIb* expression (r = -0.98) and OsSBEIIb enzyme activity (r = -0.95) in the mutants **(Supplemental Fig. 2B, C)**. Consistently, the increases in AAC are also related to the decreases in *OsSBEIIb* expression (r = -0.94) and OsSBEIIb enzyme activity (r = -0.95) in the mutants **(Supplemental Fig. 2D, E)**. Clearly, suppression of *OsSBEIIb* expression for amylopectin biosynthesis in rice leads to increased AC.

RS content and rate of reducing sugar production in indica rice cultivar TNGS14, TNG82 (WT) and seven OsSBEIIb mutant lines

The RS contents in the grains in WT and *indica* cultivar TNGS14 were very low (0.4-1.0%) but significantly increased to 6.7–7.5% in the two heterozygous and 11.6–12.0% in the five homozygous mutant lines **(**Fig. 5A**)**. Again, the increases in RS are mainly due to the decreases in *OsSBEIIb* expression (r = -0.99) and OsSBEIIb enzyme activity (r = -0.95) among the mutants **(Supplemental Fig. 2F, G)**. The rate of reducing sugar production, a gradual depletion of starch during digestion, was estimated after α-amylase treatment. The rates were much lower in the grains of the two heterozygous (Line-37, Line-64) and five homozygous *OsSBEIIb* mutant lines than that of WT **(**Fig. 5B, C**)**; and they are positively correlated to their GIs (r = 0.93) **(Table 2, Supplemental Fig. 2J)**. Again, the rates of reducing sugar production were lower in the five homozygous lines than those of two heterozygous lines and *indica* cultivar TNGS14 **(**Fig. 5B, C**)**. The decreases in the rate of reducing sugar production in the mutants are entirely due to the decreases in *OsSBEIIb* expression (r = 0.99) and OsSBEIIb enzyme activity (r = 0.96) **(Supplemental Fig. 2H, I)**.

Influences of OsSBEIIb mutations on starch viscosity

Rapid viscosity analysis (RVA) was performed with the grain flours of *indica* rice cultivar TNGS14, WT and seven CRISPR-edited mutant lines to test their resistance to alkaline gelatinization. Compared to WT, the peak viscosities (PKV) in all seven *OsSBEIIb* mutant lines decreased significantly and the varicosities for the five homozygous mutant lines were much lower than those of the two heterozygous mutant lines **(**Fig. 6**)**. Both hot viscosities (HV) and cool paste viscosities (CPV) of *OsSBEIIb* mutant lines were significantly lower than those of WT, but there were no differences between the homozygous and heterozygous mutant lines. Analogously, the starch breakdown viscosities (BDV, PKV-HV) and setback viscosities (SBV, CPV-HV) of the two heterozygous mutant lines were similar to those of WT, but much

higher than those of the five homozygous mutant lines. Whereas all these values in the seven *OsSBEIIb* mutant lines were significantly lower than those in the *indica* cultivar TNGS14, the two heterozygous mutant lines showed higher PKV and BDV values than the *indica* cultivar TNGS14 (Fig. 6). In summary, these results indicate that the structure of starch is significantly modified by down-regulation of *OsSBEIIb* in the rice endosperm that leads to changes of starch viscosity and palatability.

Discussion

Starch is the major source of carbohydrate for humans. Starch accounts for up to 75% of the mass of cereal grains (**Ordonio** et al., **2016**). Rice starch is low in RS and metabolic engineering of rice starch is mainly driven by the health benefits of RS, which has a high content of amylose and is more difficult to digest (Englyst et al., 1992). RS has been reported to provide many health benefits for humans, and high RS foods have lower glycemic and insulin responses and reduce the risk of type II diabetes, cardiovascular disease and obesity (Baxter et al., 2019). The number of death caused by diabetes increased by 80% in the past twenty years, and it is projected to increase to over 6 million people by 2040 (IDF DIABETES ATLAS Ninth edition 2019). Thus, it's important to generate high AC, high RS and low GI rice as a better source of starch for diabetes patients.

SBE is a key enzyme in the process of starch biosynthesis, forming the branched structure of amylopectin by catalysis of glucose monomer binding through α-1,6 bonds (Tian et al., 2009). *SBEIIb* deficient mutants and transgenic rice have been shown to accumulate greatly modified with as higher AC, higher RS content and higher onset temperature for gelatinization (**Nakamura** et al., **2018**). SBEIIa and SBEIIb usually produce short chains while SBEI usually produces long chains of amylopectin (Satoh et al., 2003). Down-regulating or eliminating the expression of *SBEII* in several major crops also leads to increased contents of amylose and RS. For example, editing the exon 12 or exon 18 of *OsSBEIIb* in the *japonica* cultivar Nipponbare through CRISPR/Cas9 significantly decreases its expression level and significantly increases the contents of amylose and RS in the endosperm (Baysal et al., 2020). In the present study, we developed seven *OsSBEIIb* mutant lines including both heterozygous and homozygous lines through CRISPR/Cas9 in the low amylose and low RS *japonica* rice cultivar TNG82 for molecular and physiological studies. We designed two gRNAs respectively targeting exon 3 and intron 3 of *OsSBEIIb* to generate long fragment deletion (Fig. 1A). Knock-out and knock-down of *OsSBEIIb* lead to decreased gene expression, OsSBEIIb enzyme activity and total starch content and increased amylose and RS contents. Expression of *OsSBEIIb* in homozygous lines is almost totally suppressed and is about 50% lower in heterozygous lines than that in WT (Fig. 2A). As expected, *OsSBEIIb* expression in the homozygous mutants is almost completely knocked down since rice genome contains only one copy of the gene (Yang et al., 2012). The transcript for *OsSBEI* is unaffected in *OsSBEIIb* RNAi mutant rice seed (Butardo et al., 2011; Baysal et al., 2020). Consistently, in the current work the mRNA level of *SBEI* is not affected at all in the mutant lines (**Supplemental Fig. 1**). Following the suppression of *OsSBEIIb* expression, SBE activities in heterozygous and homozygous lines are significantly reduced (Fig. 2B). The substantial SBE activity remaining in the mutant lines (Fig. 2B) is presumably due to the normal expression of other SBEs as reported previously (Butardo et al., 2011; Baysal et al., 2020).

In transgenic barley, almost all the increase in AC has been obtained by inhibiting the *SBE* genes. *HvSBEIIa* and *HvSBEIIb* activities are equal in barley endosperm, and silencing both *HvSBEIIa* and *HvSBEIIb* in barley at the same time increases AC from 28.5 to 89.3% (Regina et al., 2010). Moreover, amylose-only barley is generated when the three *SBE* genes are simultaneously suppressed (Carciofi et al., 2012). In wheat, TaSBEIIa is the major isoform of SBEII in the endosperm and RNAi inhibition of *TaSBEIIa* increases AC from 25.5% to more than 70% (Regina et al., 2006, 2015). In potatoes, AAC is increased from 22% to as high as 60–87% through small interfering RNAs (siRNA) to simultaneously inhibit the expression of *StSBEI* and *StSBEII* together (Andersson et al., 2006). The AC of ordinary maize starch is about 30% (Huang et al., 2015) and its RS content is as low as 1–2% (Chanvrier et al., 2007). ZmSBEIIb is the predominant isoform in the maize endosperm which is at least 50 times the abundance of ZmSBEIIa (Gao et al., 1997). In the maize amylose extender (*ae*) mutant AAC and RS are as high as 85.6% and 34.3%, respectively (Li et al., 2008).

OsSBEIIb is also the predominant SBEII isoform in rice endosperm, and chemical mutagenesis of *OsSBEIIb* in the *japonica* rice cultivar Jiangtangdao 1 significantly increases AC from 16.2 to 31.1% and the RS content from 0.4 to 11.7% (Yang et al., 2012). Suppression of *OsSBEIIb* expression mediated by artificial microRNA (ami-RNA) in the *japonica* cultivar Nipponbare significantly increases AC from 19.6 to 41.2% and increases RS from 0.1 to 4.4% while GI decreases from 85 to 44 (Butardo et al., 2011). Targeted editing of *OsSBEIIb* by CRISPR/Cas9 in the *japonica* cultivar Kinmaze increases its AC from 15 to 25% and RS from 0.1 to 6.5 with a 30% reduction in grain weight (Sun et al., 2017). In this study, the AAC and AC in *OsSBEIIb* homozygous mutant endosperms are 2 fold higher (36.0% and 31.8%, respectively) than WT (17.4% and 15.8%, respectively) and 1.2 fold higher than the *indica* variety TNGS14 (28.0% and 26.8%, respectively). The corresponding values in heterozygous mutant lines (25.0% AAC, 23.4% AC) are 1.44 fold and 1.48 fold higher than those of WT, respectively (Fig. 3A, B). Thus, our results are consistent with previous studies. Satoh et al., (2003) showed that *OsSBEIIb* mutant has significantly reduced weight of seeds with opaque endosperm and the starch grains are abnormal in size, shape, and distribution. The seed weight and size of our homozygous lines are also significantly decreased, but these traits are very similar between heterozygous lines and WT **(Table 2)**. The degree of chalkiness of our mutant grains is similar to that of *OsSBEIIb* mutant rice obtained in the previous study (Butardo et al., 2011).

Usually, starch with a high proportion of amylose has a high RS content. Compared with amylopectin, amylose has a smaller molecular weight, tighter arrangement and its chain structure has fewer space obstacles to regenerate crystal structure, making it difficult for α-amylase to digest (Shu et al., 2006; Gani et al., 2017). In this study we find 17.8-fold and 29.5-fold increases in RS content in the heterozygous and homozygous mutant lines, respectively, relative to WT (Fig. 4A). Expression of *OsSBEIIb* is negatively correlated with RS **(Supplemental Fig. 2F**), similar to previous studies (Butardo et al., 2011; Yang et al., 2012; Zhu et al., 2012; Sun et al., 2017).

The AC and RS contents in rice starch are negatively correlated with the rate of digestibility (i.e., release of reducing sugar during digestion) and GI (Butardo et al., 2011; Kumar et al., 2018). The higher the AC in the

grain, the lower the digestion efficiency of starch (Sajilata et al., 2006) because the hydrogen bonds that exist in the glucose chain connecting amylose molecules make it more resistant to α-amylase (Alhambra et al., 2019). The digestibility or rate of reducing sugar production in the homozygous mutant lines obtained in this study is significantly lower than those of WT and the high AC *indica* cultivar TNGS14 (Fig. 4B), due to increased AC and RS. In contrast, the rates of reducing sugar production in heterozygous mutant lines are very similar to that of *indica* cultivar TNGS14 but lower than that of the WT (Fig. 4C). This is related to the higher proportion of amylose and long-chain amylopectin and high semi-compound starch which is consisted of many individual granules held together with a smaller specific surface area. Therefore, composite particles bind less effectively to α-amylase than individual particles, which limits hydrolysis (Wei et al., 2010b; Zhu et al., 2012). In our mutant lines, the averaged GIs in heterozygous and homozygous mutant lines are 68.5 and 55.5, respectively; both are significantly lower than the GI for WT (77). The GIs of heterozygous mutant lines are similar to that of *indica* cultivar TNGS14. In spite of the fact that our homozygous mutant lines are in a *japonica* background (TNG82) that has low AC (15.8%) and the *indica* rice TNGS14 that has a high AC (26.8%), the homozygous mutant lines have a lower GI value (55.5) than WT (77) and TNGS14 (69) **(Table 2)**.

Sun et al., (2017) find significantly higher RS contents (5.2–9.8%) in their *OsSBEIIb* mutant lines with long fragment insertion and mutant lines with only 1 bp insertion at the editing site by CRISPR/Cas9. In our study, a similar RS content is found in homozygous mutant lines with long fragment insertion (Line-34, 60) and short fragment insertion (Line-33, 42, 43) as well (Fig. 4A). The levels of change in molecular and physicochemical properties in heterozygous mutant lines are almost half of those in homozygous mutant lines, indicating that the non-editing allele in heterozygous lines is being used to transcript and translate functional OsSBEIIb. Interestingly, Line-64 with deletion 211 bp and 3 bp in each allele has similar molecular and physicochemical properties to other heterozygous lines. Apparently, this allele with 3 bp deletion can lead to loss of only a single amino acid in the enzyme protein in the translation step buy does not affect the ability of the mutated OsSBEIIb to function (Fig. 1D). Comparing the target 2 (intron) mutations between Line-34 (-219/-221) and Line-60 (-219/WT), we show that the mutation on intron doesn't seem to lead to any effect on starch properties. Clearly, choosing mutant lines with high RS content, high AAC and low GI is the goal in a breeding program.

The RVA has been widely used for assessing the pasting of starch and it is often used to identify a particular characteristic of the rice variety (Kesarwani et al., 2016). The AC in rice is negatively correlated with breakdown (BDV) and peak viscosities (PKV) but positively correlated with cool paste (CPV) and setback viscosity (SBV: CPV-PKV). The low viscosity and high gelatinization temperature of high amylose rice and corn can be attributed to their high ACs (Shu et al., 1998). The swelling degree of starch is inhibited by the increase of AC. It is generally believed that starch with a low AC will show a higher viscosity and a lower gelatinization temperature (Noda et al., 2004). The high helix conformation of amylose with more hydrogen bonds requires a higher temperature to gelatinize, leading to an increase in gelatinization temperature (McGrane et al., 2004). With significantly higher AC in the endosperm starch of our *OsSBEIIb* mutant lines, their PKV, BDV and gelatinization temperature are significantly decreased but SBV is significantly increased (Fig. 5).

In this study, we further demonstrate the advantages of CRISPR/Cas9-mediated genome editing technology in breeding high-amylose rice to meet the increasing demand caused by diet-related chronic diseases such as diabetes. Compared with conventional breeding, chemical- or physical-induced mutagenesis and transgene-based strategies, CRISPR/Cas9-mediated genome editing technology can modify target genes more precisely in a very effective way (**Jones** et al., **2015)**. A previous study demonstrates that mutant lines with simultaneous repression of *OsSS IIIa* and *OsSBEIIb* in rice by RNAi has increased the expression levels of both *OsGBSSI* and *ADP-glucose pyrophosphorylase* (*OsAGPase*) genes, resulting in 1.5-fold accumulation of amylose in the endosperm compared to *OsSSIIIa* or *OsSBEIIb* single mutant line (Asai et al., 2014). Also, it has been shown that the starch synthase *SSIIa* and *SSIIIa* double mutant rice has significantly increased AC by 2-fold and increased medium-long chains of amylopectin (DP 7–11) in the *japonica* cultivar Kitaake (Zhang et al., 2011). Consistently, the double mutant *OsSBEI/OsSBEIIb* by RNAi produces high AAC (49.2%) and RS (14.9%) rice (Wei et al., 2010a). Silencing of both *OsSBEII* and *OsSBEI* at same time by antisense RNA leads to significant increase in AC from 27.2 to 64.8% and RS from 0.1 to 14.6 with a significant decrease in grain weight and size (Zhu et al., 2012). Hybridizing *OsSBEI* mutant rice (EM557) and *OsSBEIIb* mutant rice (EM10), both isolated from N-methyl-N-nitrosourea (NMU) mutagenized populations of *japonica* cultivars Taichung 65 and Kinmaze, respectively, to produce *OsSBEI/OsSBEIIb* double mutant rice also leads to much higher AAC (51.7%) and RS (35.1%) than the single mutant EM557 or EM10 (Miura et al., 2021). It's contemplated that the two SBE isoform may exhibit a synergistic effect on synthesis of amylopectin. Therefore, in the future, it has a good potential to further increase the amylose and RS contents in the *OsSBEIIb* mutant rice of this study by down-regulating the expression of *OsSSIIa*, *OsSSIIIa, OsSBEI* and *OsSBEIIb* or overexpressing *OsWxIII* at the same time through CRISP/Cas9 to provide more suitable source of starch for type II diabetes. Moreover, the transgenes used for editing can be easily eliminated from the segregating progenies (Sun et al., 2017).

Conclusion

In the present study, high AAC and RS rice with a significantly lower GI was generated through mutated *OsSBEIIb* by CRISPR/Cas9 in the *japonica* rice cultivar TNG82. We showed that the molecular and physicochemical modifications in *OsSBEIIb* homozygous and heterozygous mutant lines are significantly different from WT. The levels of modification in the molecular and physiochemical properties of starch in heterozygous mutant lines were about half as those in homozygous mutant lines. The level of gene expression and enzymic activity of *OsSBEIIb* decreased more significantly in the homozygous than heterozygous mutant lines while the AAC in the homozygous and heterozygous mutant lines were 2.1- and 1.4-fold higher than that in WT (TNG82), respectively. On the contrary, the RS contents in the homozygous and heterozygous mutant lines were 25- and 15.6-fold higher than those in WT (TNG82), respectively. With increasing RS, the GI was also progressively decreased in heterozygous and homozygous mutant endosperms. These results demonstrate that it has huge potential for precise and efficient generation of transgene-free high RS and low GI rice through CRISPR/Cas9 for the provision of a more suitable source of starch for chronic disease patients.

Abbreviations

RS: Resistant Starch

GI: Glycemic Index

SBE: Starch branching enzyme

SBEI: Starch branching enzyme I

SBEIIa: Starch branching enzyme IIa

SBEIIb: Starch branching enzyme IIb

AAC: Apparent Amylose Content

WT: Wildtype

T2DM: Type 2 diabetes

RDS: Rapidly digested starch

SDS: Slowly digested starch

AC: Amylose Content

AGPase: ADP-glucose pyrophosphorylase

NMU: N-methyl-N-nitrosourea

GT: Gelatinization Temperature

GBSSI: Granule-bound starch synthase I

SSIIa: Starch synthase IIa

SSIIIa: Starch synthase IIIa

PCR/RE: PCR-based restriction enzyme

qRT-PCR: Quantitative real-time PCR

RVA: Rapid viscosity analysis

PKV: Peak viscosities

HV: Hot viscosities

CPV: Cool paste viscosities

BDV: Breakdown viscosities

SBV: Setback viscosities

siRNA: Small interfering RNAs

ae: Amylose extender

ami-RNA: Artificial microRNA

SSF: Simulated Salivary Fluid

SGF: Simulated Gastric Fluid

SIF: Simulated Intestinal Fluid

TAME: p-toluene-sulfonyl-L-arginine methyl ester

AUC: Area under the curve

HS: Hydrolyzed starch

HI: Hydrolysis index

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Competing Interests:

The authors declare that no competing interests exist.

Availability of Data and Materials:

All relevant data are provided within the article and its supplementary information files.

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Author Contributions:

M.S.B.K, Y.-P.W. and H.-C.W. designed the research; H.-C.W. performed research; S.-Y.Y, M.S.B.K, Y.-P.W. and Y.-C.H. provided technical assistance; H.-C.W. wrote and M.S.B.K. revised the manuscript. All author(s) read and approved the final manuscript.

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Tables

Table 1. Genotype and target mutation at 2 target sites of *OsSBEIIb* in seven representative mutant lines. Target site 1 at exon 3 and Target site 2 at intron 3. The genotypes included homozygous and heterozygous mutants. Line-42, 33, 34, 43 and 60 are homozygous lines while Line-37 and 64 are heterozygous at target site 1 (exon 3).

Line number	Genotype (Phenotype)	Target 1 (exon 3) Allele 1 / Allele 2	Target 2 (intron 3) Allele 1 / Allele 2
Line-42	Homozygous	-5 / -5	+1 / +1
Line -33	Bi-allele (Homo)	-19 / -8	+1 / +2
Line -43	Bi-allele (Homo)	+1 / -2	-29 / -28,+90
Line -34	Bi-allele (Homo)	-219 / -221	-219 / -221
Line -60	Bi-allele (Homo)	-219 / +210	-219 / WT
Line -64	Bi-allele (Hetero)	-3 / -221	-221 / +1
Line -37	Heterozygous	WT / -26	+1 / +1

Table 2. Comparison of grain morphology and starch physiochemical properties of representative *OsSBEIIb* mutant lines with their WT (TNG82) and TNGS14. *Mean values ± SD with different letters are significantly different* (t-test, 3 replicates of samples, b: $P < 0.05$, c: $P < 0.01$). *Descriptions indicated in the brackets under each OsSBEIIb mutant lines: d or WT represents deletion of nucleotides at the first target site or wild type genotype.*

Properties	TNGS14 (*indica*)	TNG82 (WT)	Line-34 (d219/d221)	Line-42 (d5/d5)	Line-37 (WT/d26)	Line-64 (d3/d221)
Hundred-grain weight (g)	$2.6^{b} \pm 0.3$	$2.8^{a} \pm 0.1$	$2.1^{c} \pm 0.1$	$1.9^{c} \pm 0.3$	$2.8^{a} \pm 0.1$	$2.8^{a} \pm 0.1$
Length (mm)	$6.7^{a} \pm 0.06$	$5.8^{b} \pm 0.1$	$5.4^{c} \pm 0.2$	$5.4^{c} \pm 0.2$	$5.9^{b} \pm 0.2$	$5.8^{b} \pm 0.1$
Width (mm)	$2.16^{c} \pm 0.1$	$3.2^{a} \pm 0.1$	$2.8^{b} \pm 0.2$	$2.9^{b} \pm 0.1$	$3.1^{a} \pm 0.1$	$3.0^{a} \pm 0.1$
Thickness (mm)	$2.0^{b} \pm 0.2$	$2.3^{a} \pm 0.1$	$1.8^{c} \pm 0.1$	$1.9^{c} \pm 0.2$	$2.0^{a} \pm 0.1$	$2.1^{a} \pm 0.1$
Chalkiness (% per grain)	0-20	0-10	90-100	90-100	10-25	10-25
Onset gelatinization temperature (°C)	76.9^{b}	69.7^{a}	80.1^{c}	79.5^{c}	71.5^{a}	71.0^{a}
Predicted glycemic index	$69^{b} \pm 0.6$	$77^{a} \pm 2$	$56^{c} \pm 1.4$	$55^{c} \pm 0.7$	$69^{b} \pm 0.3$	$68^{b} \pm 1$

Figures

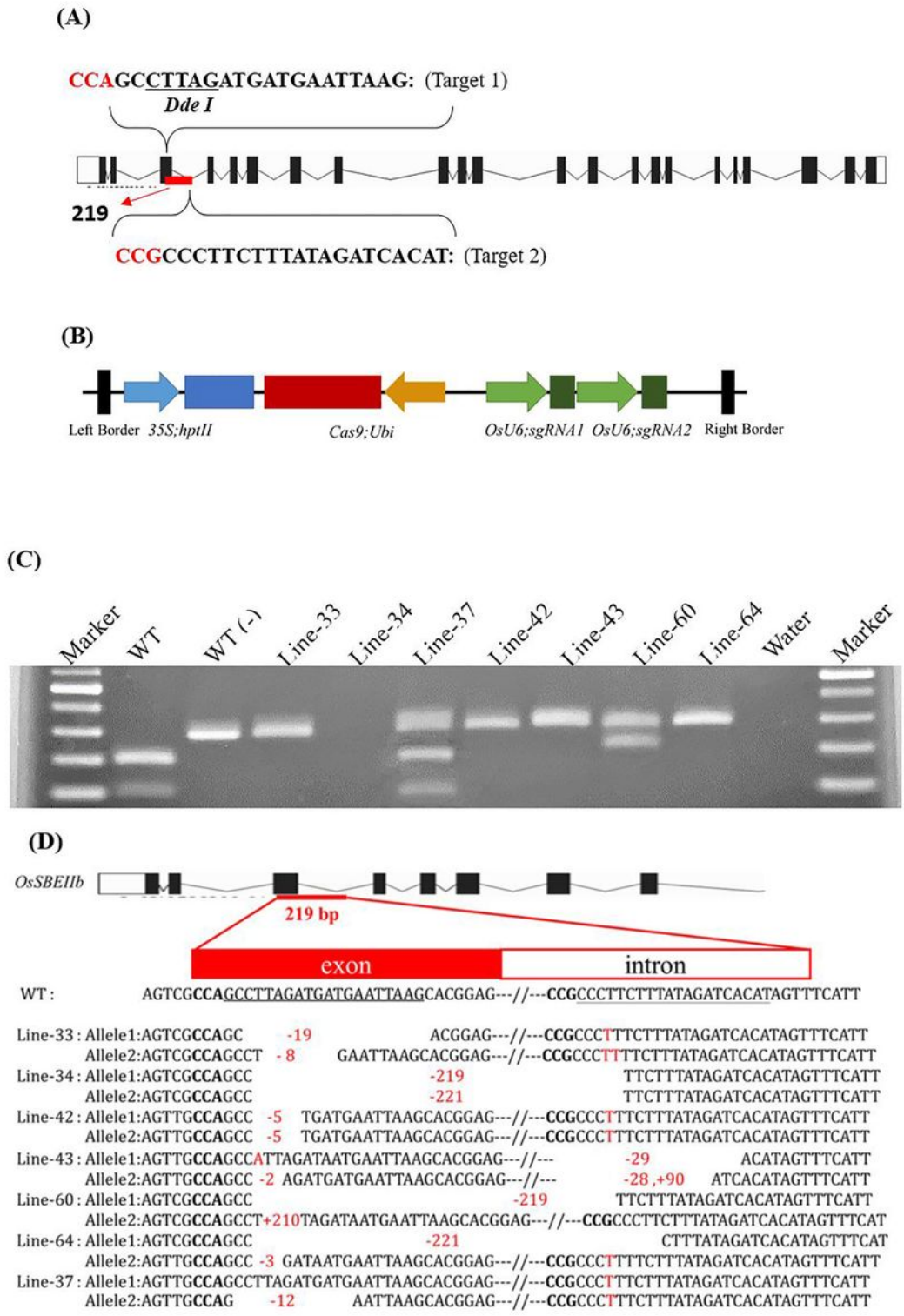

Figure 1

CRISPR/Cas9 induced mutations in OsSBEIIb. (A) A schematic map of the gRNA target sites on the genomic regions of OsSBEIIb. Exons are shown as boxes; introns are shown as lines; the recognition sites of the enzyme Dde I are underlined; and the protospacer adjacent motif (PAM) is shown in red. (B) The T-DNA structure in the CRISPR/Cas9-mediated genome editing construct. (C) Detection of first target site mutations in OsSBEIIb via PCR/RE assay in T0 mutants. The PCR products of OsSBEIIb mutant lines are

resistant to digestion of Dde I. '-' means the PCR products of WT DNA undigested by Dde I; '+' means digestion. (D) Sequence analyses of the seven OsSBEIIb mutant lines. The PAM motifs are in boldface, target sequences are underlined and insertions and deletions are highlighted in red.

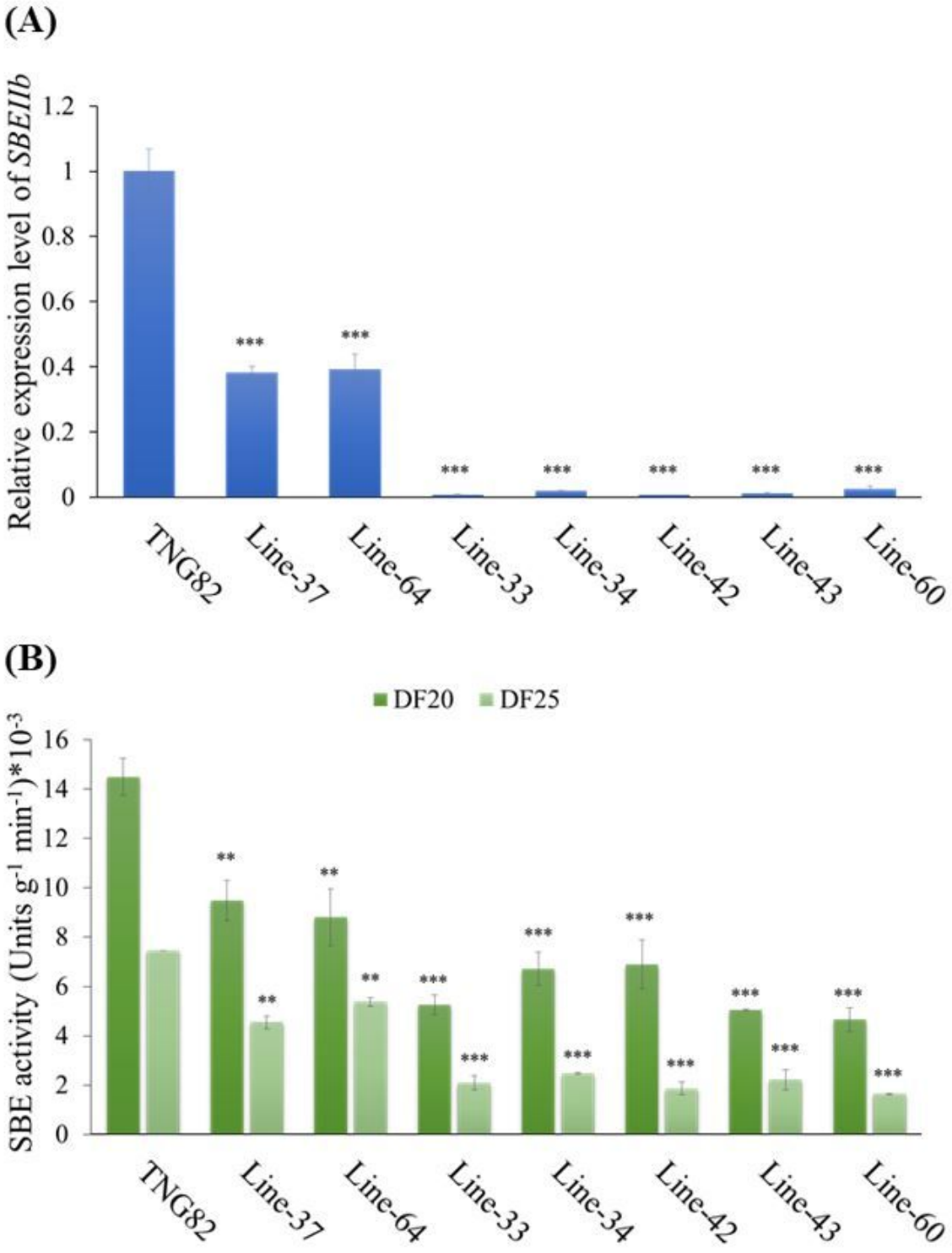

Figure 2

Gene expression levels and enzyme activities of TNG82 (WT) and seven OsSBEIIb mutant lines. (A) Expression levels of OsSBEIIb in the endosperms of WT, and representative heterozygous and

homozygous mutant rice lines, as analyzed by qRT-PCR. Total RNA was isolated from young endosperms 25 days after flowering. The expression level of ribosomal RNA was used as an internal control for normalization. The expression level of the japonica TNG82 was included as a reference. (B) Enzyme activities of SBE in the endosperms during rice grain filling of TNG82 and OsSBEIIb mutant lines. Total soluble protein was isolated from fresh endosperms 20 and 25 days after flowering. Error bars indicate SD of means (t-test, 3 replicates of endosperm samples, ** P < 0.05, *** P < 0.01).

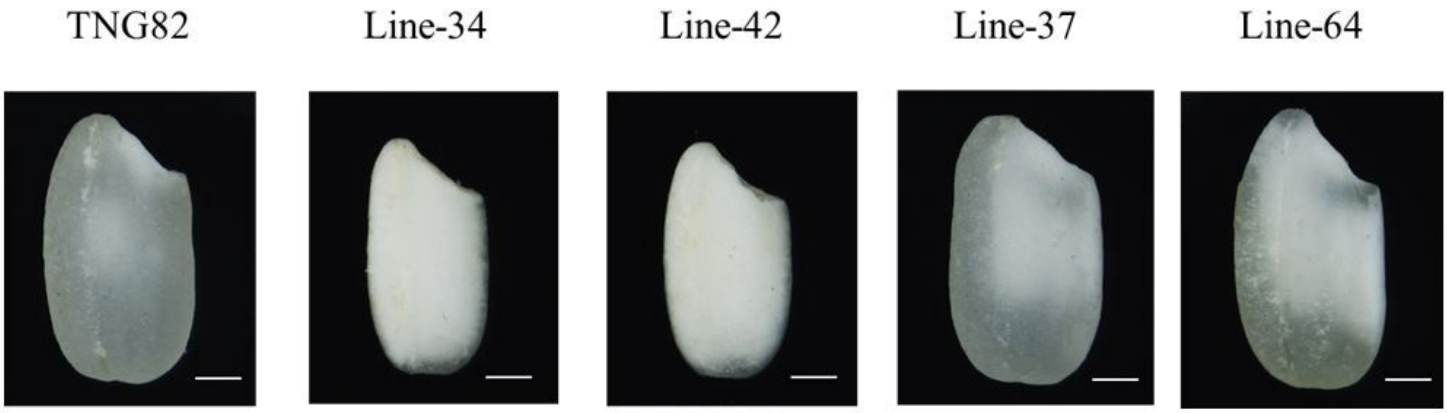

Figure 3

Grain gross morphology of TNG82 (WT), homozygous mutant lines (Line-34, 42), and heterozygous mutant lines (Line-37, 64) at maturity. (Scale bars, 1 mm).

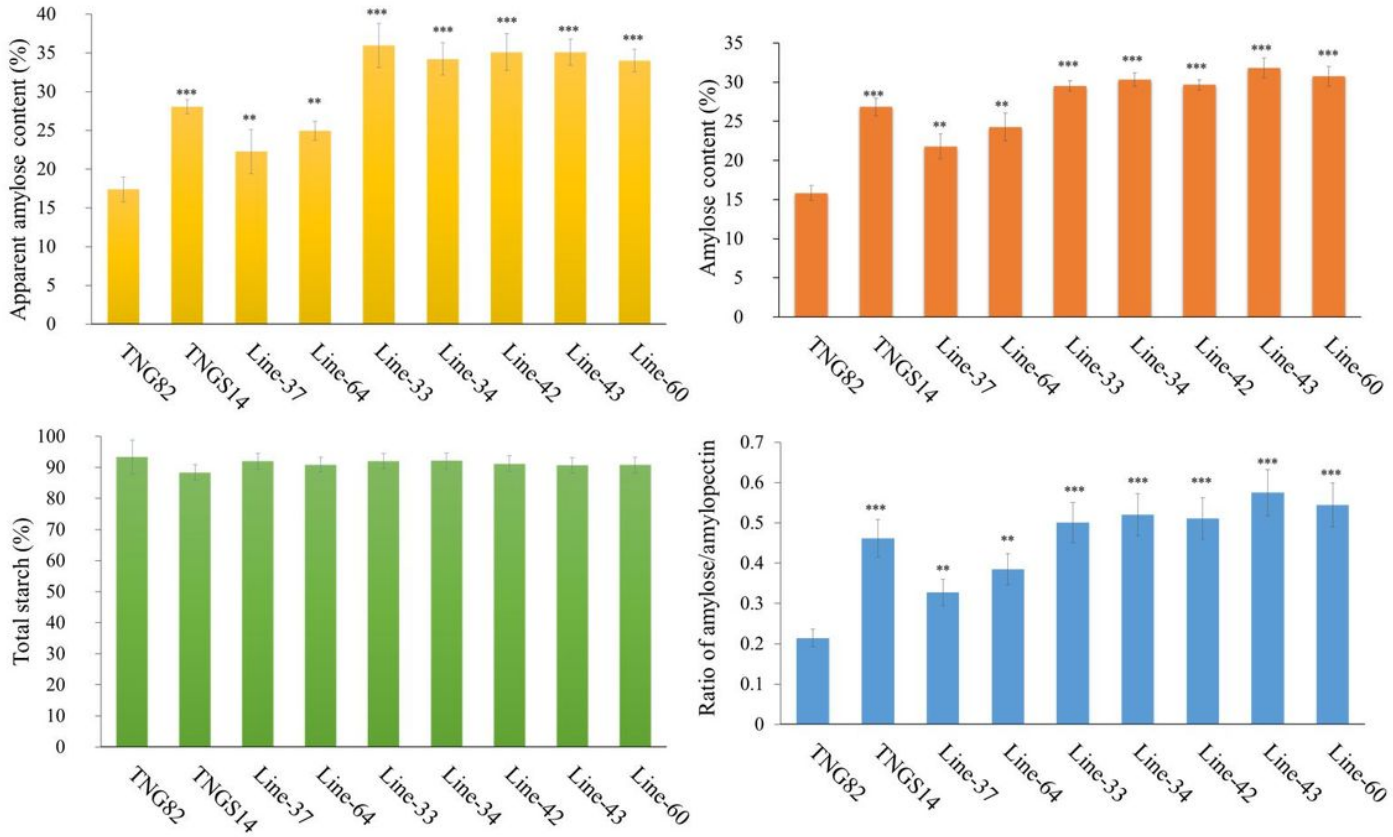

Figure 4

Starch contents of polished grains of TNG82 (WT), TNGS14 and seven OsSBEIIb mutant lines. (A) Apparent amylose contents (AAC) of polished grains. (B) Amylose contents (AC) of polished grains. (C) Total starch contents of polished grains. (D) Ratios of amylose/amylopectin of starch. Error bars indicate SD of means (t-test, 3 replicates of samples, ** P < 0.01, *** P < 0.001).

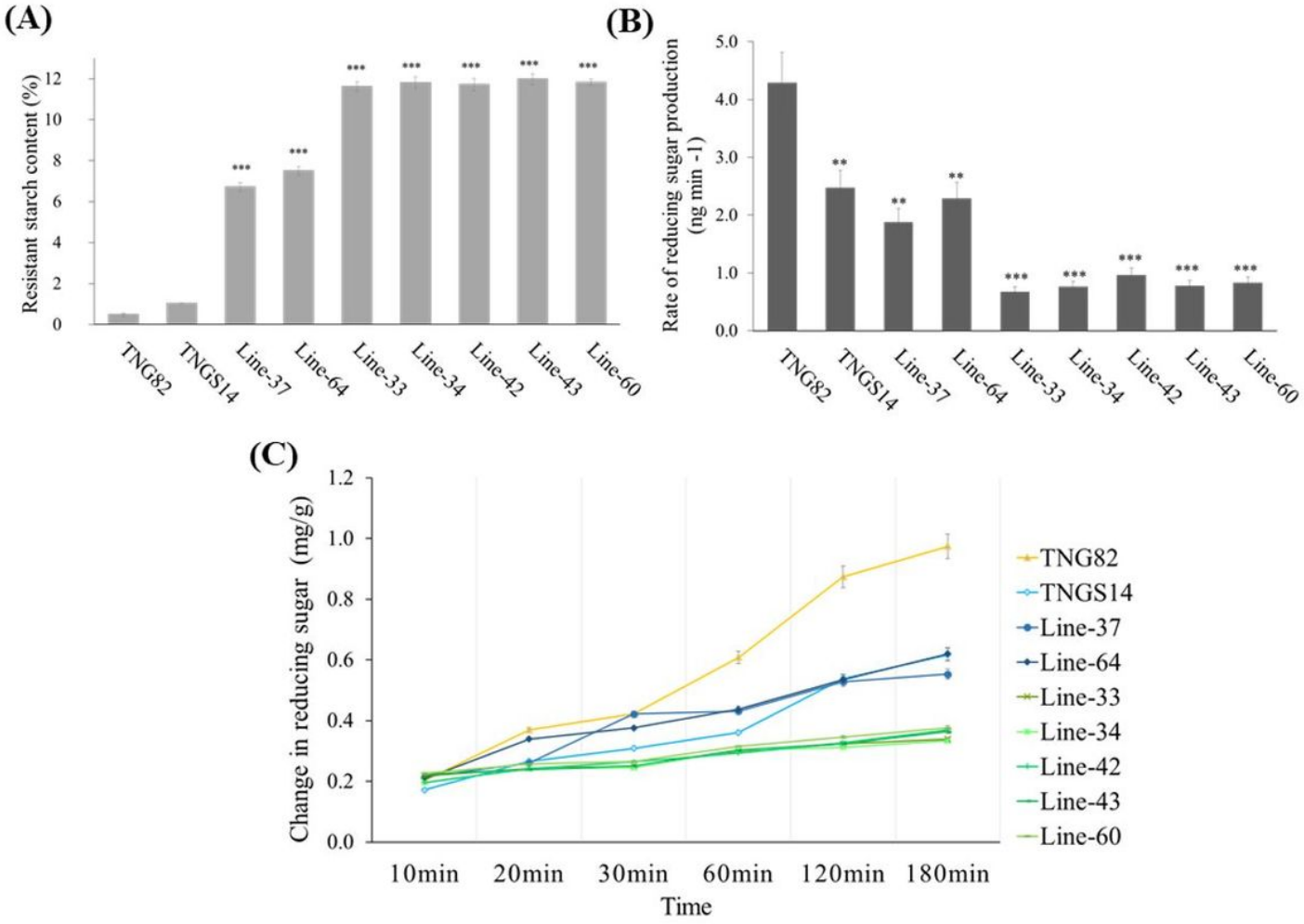

Figure 5

RS contents and rates of reducing sugar production during starch digestion in polished grains of TNG82 (WT), TNGS14 and seven OsSBEIIb mutant lines. (A) RS contents. (B) Rates of reducing sugar production. (C) Changes in reducing sugar after α-amylase treatment. Error bars indicate SD of means (t-test, 3 replicates of samples, ** P < 0.005, *** P < 0.001).

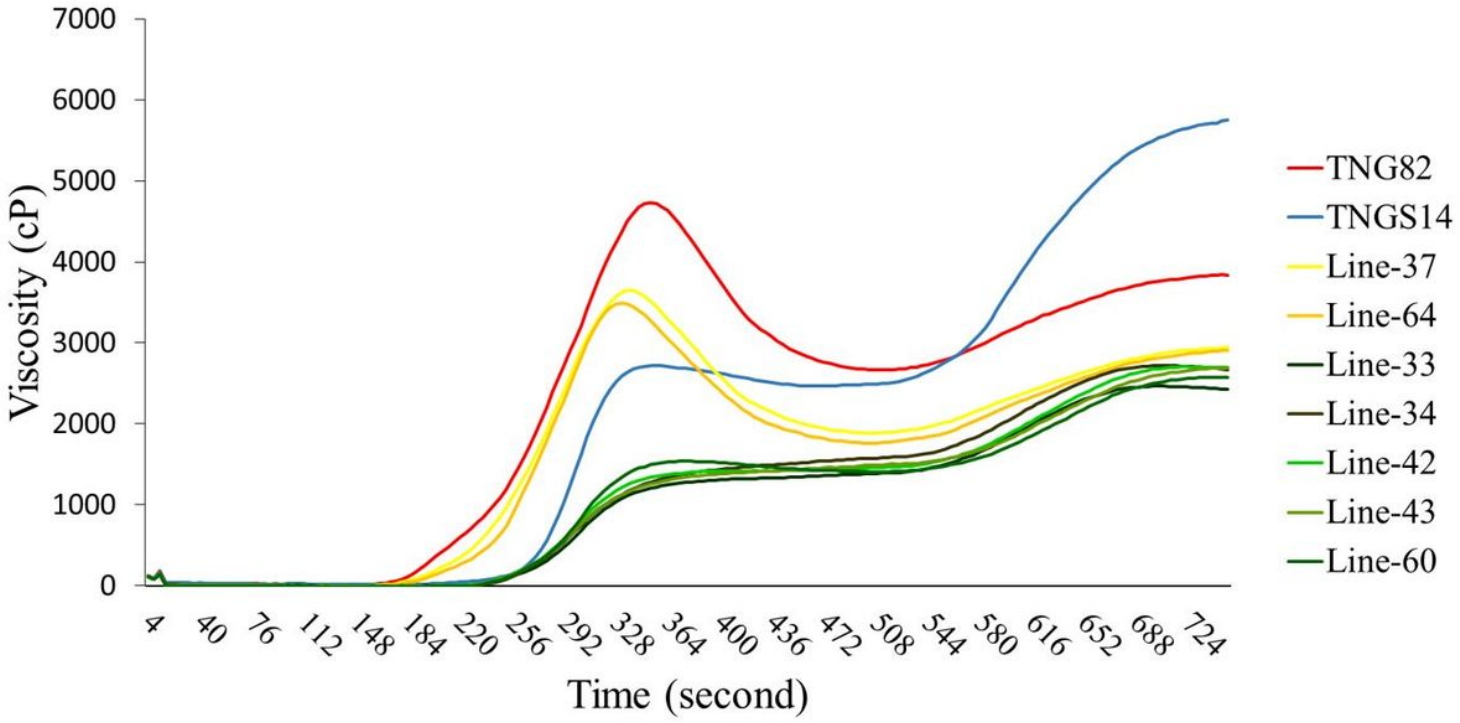

Figure 6

Rapid viscosity profiles of rice flours in TNG82 (WT), TNGS14 and seven SBEIIb mutant lines. Pasting curves were measured by a rapid visco analyser (RVA). PKV, peak viscosity; HV, hot viscosity; CPV, cool paste viscosity.

Supplementary Files

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