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Research Article

Efficient Production of High 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. Rice starch is low in Resistant Starch (RS) with a high Glycemic Index (GI). RS has gained importance since it is beneficial in preventing various diseases. Starch Branching Enzyme *IIb* (*SBEIIb*) plays a key role in amylopectin synthesis in the endosperm of cereals. In this study, we mutated *OsSBEIIb* in the *Japonica rice* cultivar Tainung82 (TNG82) through CRISPR/Cas9 and investigated the molecular and physicochemical modifications in *OsSBEIIb* mutant lines, e.g., gene expression, enzyme activity, Amylose Content (AC), RS and GI. As expected, gene expression and enzyme activity of *OsSBEIIb* were down-regulated significantly while AC and RS contents increased progressively from 17.4% and 0.5% in weight, 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. Transgene-free plants were subsequently identified in the T1 populations. Our results demonstrate the precise and efficient generation of high RS and low GI transgene-free rice through CRISPR/Cas9 to provide a more suitable source of starch for type II diabetes.

Keywords: Rice; *SBEIIb*; CRISPR/Cas9; Amylose; Resistant starch; Glycemic index

INTRODUCTION

Diabetes mellitus is a chronic disease and Type 2 Diabetes Mellitus (T2DM) is the most common form, representing 90% to 95% of diagnosed diabetes cases [1]. 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 [2]. 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 [3].

Starch is the main carbohydrate in human diet and the main source of energy, and 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 RS has a lower GI value. RS refers to starch that cannot be digested and absorbed in the human small intestine [4]. RS naturally exists in starchy foods like rice, corn and potatoes, and can be divided into five subtypes. Type 1 RS is synthesized in the endosperm of cereal grains or seeds with an anti-digestive effect due to its surrounding by protein matrix and cell wall material. Type 2 is found in some starchy foods (e.g., raw potatoes and immature green bananas), which display the B or C-type polymorph and are highly resistant to enzymatic hydrolysis. Type 3 is formed when certain starchy foods are cooked and cooled. After cooling, amylose and long branch chains of amylopectin form double helices which cannot fit into the binding site of amylase and hydrolyzed. Type 4 is a chemically modified starch, formed by cross- linking or by adding chemical derivatives, such as octenyl succinic groups to change the structure of the starch and become partially resistant to enzymatic hydrolysis. Type 5 is an amylose-long branch chain of amylopectin-lipid single-helical complexes that can restrict the enzyme hydrolysis [5]. RS functions

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similarly to soluble fiber; it helps feed the probiotic bacteria in gut and increases the production of short-chain fatty acids such as butyrate [6]. 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 90% of the total mass of rice seeds [7]. Structurally, starch can be divided into amylose and amylopectin. Amylose is consisted of glucose residues linked by α (1→4) glycosidic bonds and form long linear chains with a Degree of Polymerization (DP) <5000. 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 [8]. 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 W_x locus in the rice genome which encodes the Granule- Bound Starch Synthase I (GBSSI) protein. W_x has three major W_x alleles, $W_{x\text{-}I}$, $W_{x\text{-}II}$ and $W_{x\text{-}III}$. $W_{x\text{-}I}$ bears a loss-of- function mutation that results in glutinous rice varieties with extremely low AC (<2%), $W_{x\text{-}II}$ 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 [9]. *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 for synthesis of long chains of amylopectin with DP>30. Down-regulation of *OsSSIIa* or *OsSSIIIa* significantly increases the expression level of *OsGBSSI*, Amylose Content (AC) but decreases the amylopectin content in rice [10,11].

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 from amylose and pass the broken short chain for reconnection to the acceptor chain through α (1→6) glycosidic bonds to form amylopectin . There are two classes of *SBE*s such as *SBEI* and *SBEII*. *SBEI* is responsible for the synthesis of short-chain branching of amylopectin while SBE*II* is responsible for the synthesis of long-chain branching of amylopectin. In the endosperm of monocots, *SBEII* is divided in *SBEIIa* and *SBEIIb* [12]. In rice and maize endosperms, *SBEIIb* is the major isoform [13]. The amount of *SBEIIa* is 2-3 fold higher than *SBEIIb* in wheat endosperm while the activity of *SBEIIa* is equal to that of *SBEIIb* in barley endosperm [14,15].

Among most cereals, amylose is the main linear component of starch and usually accounts for 15%-25% [16]. The AC of wild and cultivated rice ranges from 0% to 30% as affected by W_x genes. Overexpression of the appropriate W_x gene which encodes the *GBSSI* can further increase the level of amylose while down-regulation of the expression of the enzymes involved in the biosynthesis of amylopectin such as *SBEI* and *SBEIIb* thereby reduces the synthesis of amylopectin and increases the proportion of amylose and RS in cereals and potatoes [17-20].

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 [21]. Compared with WT, the endosperm of the transgenic rice with suppressed expression of *OsSBEIIb* gene by CRISPR/Cas9 has a higher RS, AC and gelatinization temperature, and stronger resistance to enzymatic hydrolysis [22]. Starch granules with a higher ratio of long chains of amylopectin are more resistant to gelatinization. Consistently, deletion or suppression of *OsSBEIIb* by chemical mutagenesis, RNAi and CRISPR/Cas9 targeted editing in rice causes a significant increase in RS and AC and a significant decrease in short side-chain branching of amylopectin significant increase of AC also changes starch chemical properties, e.g. decreased swelling power and viscosity or increased gelatinization temperature [23,24].

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 [25]. A positive relationship between RS and amylopectin long branches has been established in previous studies [26,27]. Generating high RS crops through inhibition of the *SBEIIb* gene leads to a strong anti- digestive property, with beneficial effect to human health. A large number of high RS crops have been generated through down-regulation of *SBE* gene by chemical mutagenesis, RNA interference (RNAi) and CRISPR/Cas9, including high RS and amylose wheat, rice, maize, barley and potato [28-31]. 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 time-consuming screening, low efficiency and cannot keep pace with the demand for increased crop production. On the other hand, genetic engineering provides an alternative to increase crop productivity by overcoming these limitations [32]. 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 [33]. Thus, CRISPR/Cas9-mediated gene editing technology has the potential to greatly facilitate plant breeding. For example, CRISPR/Cas9-mediated loss-of-function mutations of the starch branching enzymes *SBEI* and *SBEIIb* in rice leads to higher RS and AC. Targeted Mutagenesis of *TaSBEIIa* by CRISPR/Cas9 in both winter and spring wheat varieties was used to generate transgene-free high RS wheat with significantly increased AC.

In this study, we report the feasibility of creating high AC, high RS and low GI rice plants through CRISPR/Cas9- mediated target gene editing of *OsSBEIIb* in the *Japonica rice* cultivar Tainung82 (TNG82). CRISPR/Cas9 has advantages over other means of gene editing, including low cost, easy operation, and high efficiency. 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, RS, AC, 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 single guide RNA (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 *BsaI* site of the vector pYLCRISPR/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 pCAMBIA1300 (CAMBIA, Canberra, Australia) [34]. The sgRNA and off target site analysis were designed by CRISPR-P 2.0 design tool [35]. 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 [35,36]. 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 pYLCRISPR/Cas9Pubi-H and incubated at 28°C in dark for 3-4 days. After co-cultivation, calli were thoroughly washed with 250 mgL^{-1} cefotaxime in sterile distilled water and transferred to a selection medium containing 50 mgL^{-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 2.5mgL^{-1} kinetin, 1 mgL^{-1} NAA and 50 mgL^{-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 Wild Type (WT) plants, as described [37]. 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 Polymerase Chain Reaction (PCR) analysis 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 *DdeI*; 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 Deoxy ribonucleic Acid (DNA) in PCR reaction are 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 and 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 were 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 Os*SBEIIb* gene expression by qRT-PCR

Total RNA was isolated from endosperm tissues [34]. 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 and M-MLV reverse transcriptase (Promega) [35]. Two μg DNA was used as a template for gene expression assay by qRT-PCR; and the protocols of Livak and Schmittgen were followed using specific primers qcSBE3- F2/qc*SBE3-R2* for *OsSBEIIb* and primers qcSBE1-F1/qcSBE1-R1 for *OsSBEI*. Expression of 17S rRNA (accession number X00755) was used as an internal control for normalization of expression levels [36,37].

Activity assay of Starch Branching Enzyme (SBE)

The method for assaying the total activity of *SBE* in rice grains includes the activities of *SBE1*, *SBEIIa*, and *SBEIIb* together as follows [38].

Analysis of Apparent Amylose Content (AAC)

AAC was determined by the standardized protocol ISO 6647-1 [39].

Analysis of total starch, amylose content, resistant starch and amylopectin content

The total starch, AC and RS content in the rice flour were measured *in vitro* with the starch assay kits Megazyme K- STAR, K-AMYL and K-RSTAR, 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 [40]. 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 is slightly modified for viscosity

analysis. Three grams 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°C for 1 min, the temperature was increased to 95°C at a rate of 12°C/min and maintained at 95°C for 2.5 min. Then the temperature was reduced to 50°C at 12°C/min and maintained at 50°C 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 fast viscosity characteristic values:

Peak Viscosity (PKV): The first high viscosity after the starch solution thickens.

Hot Paste Viscosity (HPV): The lowest point of viscosity after starch is completely gelatinized.

Cool Paste Viscosity (CPV): The high viscosity produced by the heated starch solution after cooling, also known as final viscosity.

Breakdown Viscosity (BDV): PKV-HPV (Peak Viscosity-Hot Paste Viscosity).

Setback Viscosity (SBV): CPV-PKV (Cool Paste Viscosity-Peak Viscosity).

Measurement of glycemic index through static *in vitro* digestion

The consensus INFOGEST protocol was followed for the static *in vitro* digestion [41]. The obtained values of starch available were normalized to percentage of hydrolyzed starch.

Calculation of glycemic index

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) [42]. AUC of total starch hydrolyzed during the digestion was calculated manually as the sum (0-240 min) of areas between sample points, considering the area of the trapezium formed by the points in the graph Equation:

$$AUC_{2-30}=(\%HS_2+\%HS_{30})/2 \times t_{2-30} \quad (1)$$

Where AUC_{2-30} represents the area between 2 min and 30 min, HS_2 represents the percentage of total starch hydrolysed 2 min after, HS_{30} represents the percentage of total starch hydrolysed after 30 min and t represents the time between 2 and 30 min. The AUCs of both sample and reference food (pure potato starch; 121096.1211 Panreac, Spain) were used to calculate the Hydrolysis Index (HI) [43], according to Equation:

$$HI=(AUC_{sample}/AUC_{ref}) \times 100 \quad (2)$$

Where AUC sample corresponds to the AUC of the rice sample and AUC of the reference corresponds to the AUC of the reference food. GI was then determined according to Equation:

$$GI=39.71+(0.549 \times HI) \quad (3)$$

Data analyses

Grain morphology, starch molecular and physiochemical properties of TNG82 (untransformed wild type), TNS14 (*indica* rice variety) and *OsSBEIIb* mutant lines were Analysis of Variance (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* and production of transgenic rice 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. Target 1 sequence contains a restriction site for screening of mutations using PCR-based restriction enzyme (PCR/RE) digestion. The CRISPR/Cas9 construct for rice transformation contains two gRNA cassettes in the vector pYLCRISPR/Cas9Pubi-H. 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 [44]. In total, 15 independent *OsSBEIIb* mutant rice lines were selected from 46 hygromycin resistant transgenic calli through PCR/RE assay for detailed analysis. These mutant lines exhibited normal growth phenotype. 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*. The target sites on both alleles of *OsSBEIIb* in Line-34 had long deletion mutations that primer (*SBE3-R2*) has no biding site; therefore, there was no PCR product generated by the primer set (*SBE3-F2*, *SBE3-R2*) in Line-34.

Thus, 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 as shown in Table 1. However, some mutant's harbored large insertions, e.g., Line-60 had 210 bp insertions at the first target site that contains *a DdeI* cutting site. In addition, there were several short insertion and deletion mutants with 1-29 bp (Line-33, 37, 42, 43, 64). Line-64 is considered as heterozygous for phenotype because it has 221 bp deletion at one allele and 3 bp deletion at the other allele of Target 1, which may not change *OsSBEIIb* function. Representative *OsSBEIIb* homozygous and heterozygous mutant lines were chosen for comparison of with WT and *indica* cultivar TNGS14 for various starch-related traits. 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 are a powerful new tool in biology, but the on-target activity and off-target effects of individual sgRNAs can vary widely. Therefore, 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 (Table 1).

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, 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) relative 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 [45]. In contrast, the expression levels of Os*SBEI* were not altered in these mutant lines. The total enzyme activities of *SBE* in young rice grains 20 and 25 days after flowering were compared between WT and mutant lines. 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. when 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). The substantial activities remaining in the mutant grains are presumably due to the normal expression of other *SBE*s (Figure 1) [46].

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 phenotypes included homozygous and heterozygous mutants. Line-42, 33, 34, 43 and 60 are homozygous lines while Line-37 and 64 are heterozygous lines.

Line number	Genotype	Phenotype	Target 1 (exon 3)	Target 2 (intron 3)
			Allele 1/Allele 2	Allele 1/Allele 2
Line-42	Bi-allele (Homo)	Homozygous	1	1
Line-33	Bi-allele (Hetero)	Homozygous	2.375	0.5
Line-43	Bi-allele (Hetero)	Homozygous	-0.5	-29/-28,+90
Line-34	Bi-allele (Hetero)	Homozygous	0.990950226	0.990950226
Line-60	Bi-allele (Hetero)	Homozygous	-1.042857143	-219/WT
Line-64	Bi-allele (Hetero)	Heterozygous	73.66666667	-221
Line-37	Mono-allele	Heterozygous	WT/-13	1

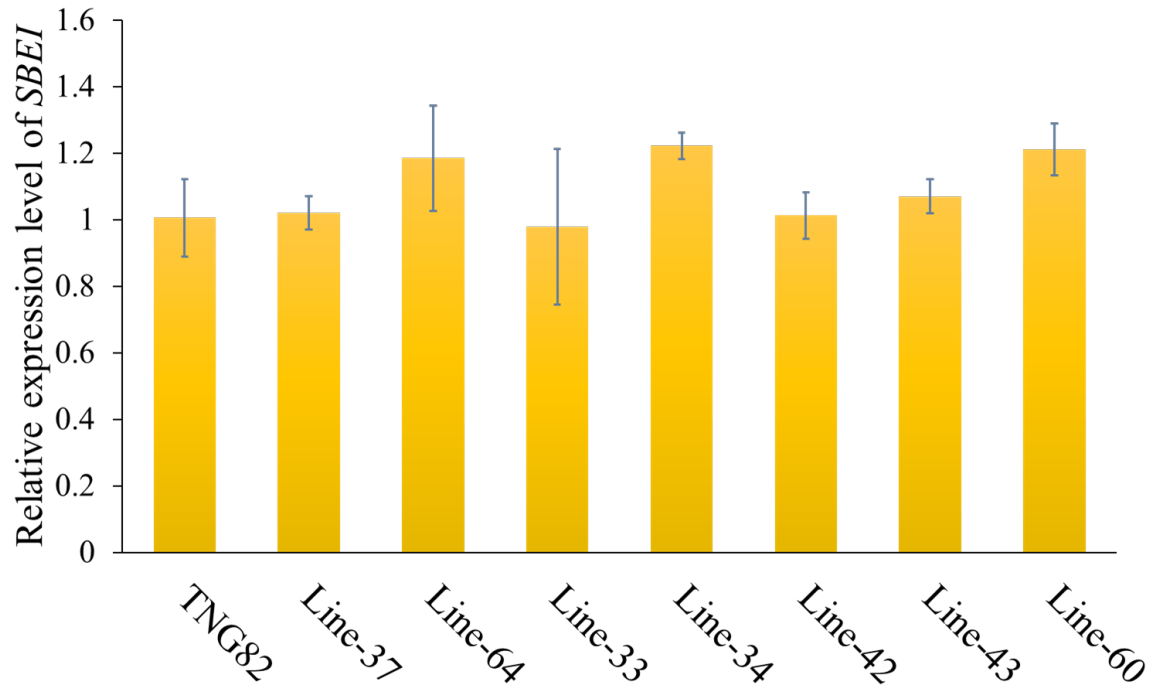

Figure 1: Expression levels of *SBEI* in the endosperms of TNG82 (WT) and representative heterozygous and homozygous mutant rice lines, as analyzed by qRT-PCR. Total RNA was isolated from young endosperms 25 days after flowering. The expression level of 17S ribosomal RNA was used as an internal control for normalization.

Grain morphology and starch physicochemical properties

WT, an *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 bp deletion at each allele at the first target site were selected for comparison of their grain morphology and starch physiochemical properties. The grain weight (68.5%-74.4%), length (90.7%-92.2%), width (79.1%-81.6%) and thickness (64.9%-68.9%) 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. 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. 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 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 (Figures 2A and 2B and Table 2).

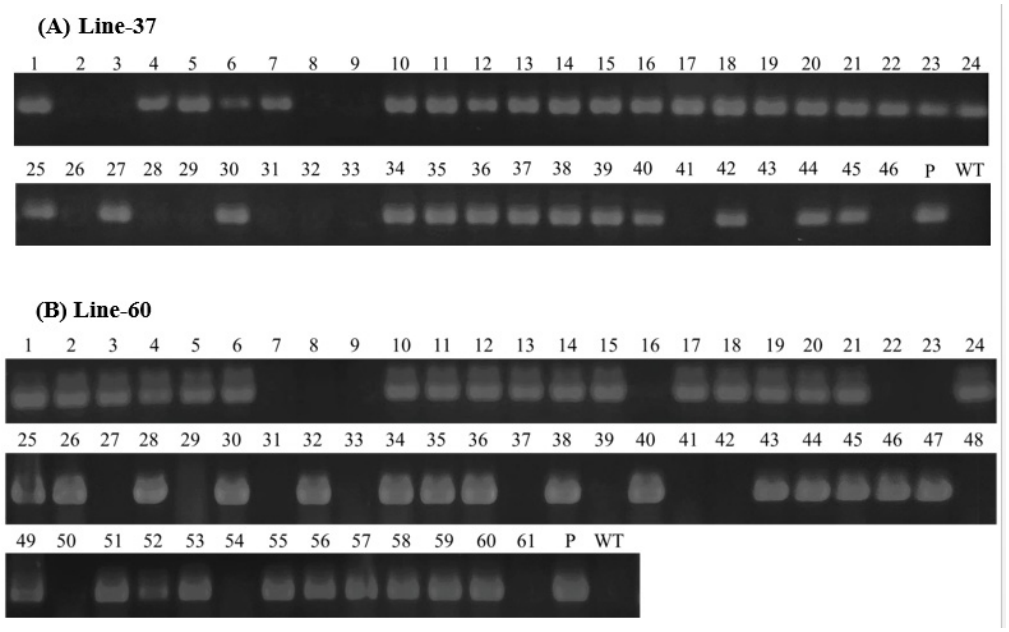

Figure 2: Screening of transgene-free T1 transgenic rice plants from Line-37. (A): Line-60; (B) by PCR of the selection marker *hptII*. Self-pollinated T1 seeds were germinated in water and genomic DNA extracted from one-week-old plants was used for PCR. *hptII* PCR fragment was not detected in 13 out of 46 of Line-37 T1 plants (# 2, 3, 8, 9, 26, 28, 29,31, 32, 33, 41, 43 and 46); (B): In 18 out of T1 plants of Line-60 (#7, 8, 9, 16, 22, 23, 27, 29, 31,33, 37, 39, 41, 42, 48, 50, 54 and 61). **Note:** P: pYLCRISPR/Cas9Pubi-H plasmid DNA, WT: Wild Type DNA (control).

Table 2: Comparison of grain morphology and starch physiochemical properties of representative *OsSBEIIb* mutant lines with their WT (TNG82) and TNGS14 (*indica*). Mean values ± SD with different letters are significantly different. Descriptions indicated in the brackets under each *OsSBEIIb* mutant lines or WT represents deletion of nucleotides at the first target site or wild type genotype.

Properties	TNG82(Wild type)	TNGS14(*indica*)	Line-34(-219/-221)	Line-42(-5/-5)	Line-37(WT/d-6)	Line-64(-3/-221)
Hundred-grain weight (g)	2.86 ± 0.1(100%)	2.67 ± 0.3	2.13 ± 0.1(74.4%)	1.96 ± 0.3(68.5%)	2.80 ± 0.1(97.9%)	2.78 ± 0.1(97.2%)
Length (mm)	5.70 ± 0.089(100%)	6.78 ± 0.06	5.26 ± 0.02(92.20%)	5.17 ± 0.16(90.70%)	5.73 ± 0.05(100.10%)	5.61 ± 0.05(98.20%
Width (mm)	3.21 ± 0.04(100%)	2.16 ± 0.02	2.54 ± 0.07(79.10%)	2.62 ± 0.07(81.6%)	3.03 ± 0.02(94.40%)	3.05 ± 0.06(95.00%)
Thickness (mm)	2.25 ± 0.02(100%)	2.00 ± 0.02	1.55 ± 0.04(68.90%)	1.46 ± 0.03(64.90%)	2.17 ± 0.05(96.40%)	2.13 ± 0.02(94.60%)
Chalkiness(% per grain)	0-10	0-20	90-100	90-100	25	25
Onset gelatinization temperature (°C)	69.7	76.9	80.1	79.5	71.5	71
Predicted Glycemic Index (GI)	77± 2	69 ± 0.6	56 ± 1.4	55 ± 0.7	69 ± 0.3	68 ± 1

Resistant starch, amylose content, total starch content and ratio of amylopectin/amylose 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. The increases in RS in the mutants are mainly due to the decreases in *OsSBEIIb* expression (r=-0.99) and *OsSBEIIb* enzyme activity (r=-0.95).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. 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. 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. 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. Taken together, these results clearly show that a total knockout of *OsSBEIIb* in the rice increases AC from 15.8 to 31%-35% in the mutants of TNG82, mainly due to the decreases in *OsSBEIIb* expression (r=-0.98) and *OsSBEIIb* enzyme activity (r=-0.95) in the mutants. Consistently, the increases in AAC are also related to the decreases in *OsSBEIIb* expression (r=-0.94) and *OsSBEIIb* enzyme activity (r=-0.95). Clearly, suppression of *OsSBEIIb* expression for amylopectin biosynthesis in rice leads to increased AC and RS contents.

Rate of reducing sugar production in indica rice cultivar TNGS14, TNG82 (WT) and seven Os*SBEIIb* mutant lines

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-37,64) and five homozygous *OsSBEIIb* mutant lines than that of WT and they are positively correlated to their GIs among these plants (r=+0.93) and again, the rates of reducing sugar production were lower in the five homozygous lines than those of the two heterozygous lines and *indica* cultivar TNGS14.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).

Influences of Os*SBEIIb* 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 viscosity and gelatinization temperature. Compared to WT, the Peak Viscosity (PKV) in all seven *OsSBEIIb* mutant lines decreased significantly and the viscosities for the five homozygous mutant lines were much lower than those of the two heterozygous mutant lines. 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 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.

Selection of transgene-free T1 transgenic plants II

Based on preliminary segregation analysis of resistance of T1 seeds to germinate on hygromycin-containing medium, we selected two transgenic lines (Line-37,60) with one copy of transgene in the rice genome, for screening of transgene- free T1 transgene plants. Line-37 is a mono-allelic mutant with 13 bp deletion on one allele (WT/-13) whereas Line-60 is a bi-allelic mutant with deletion of 219 bp on one allele and addition of 210 bp in another allele (-219/+210). A total of 46 (Line-37) and 61 (Line-60) seedlings were germinated in water and subjected to PCR screening of the selection marker *hptII*. The results showed 13 out of 46 Line-37 T1 plants (1:3.5) and 18 out of 61 Line-60 T1 plants (1:3.4) contain no transgene. The segregation ratios in *hptII* for both lines are consistent with an insertion copy of the CRISPR-Cas9 transformation construct in their genome. The *SBEIIb* mutants of these transgene-free plants were subject to PCR/RE analysis and gene sequencing to confirm the editing. From the 13 transgene- free T1 plants of Line-373 heterozygous mutant plants and 10 WT plants were found and from the 18 transgene-free T1 plants of Line-60 6 homozygous mutant plants (one -219/-219, five +210/+210) and 12 bi-allelic mutant plants (-219/+210) were identified. PCR/RE analysis of 14 transgene-containing T1 plants of Line-37 also showed 5 heterozygous mutant plants and 9 WT plants (data not show). Taken together, these results demonstrated the ease to eliminate the CRISPR-Cas9 transgene from the progenies while retaining the gene editing.

DISCUSSION

Rice starch is low in RS and metabolic engineering of rice starch is mainly driven by the health benefits of RS which is more difficult to digest [47]. RS has been reported to provide many health benefits for humans, and high RS foods have l ower glycemic and insulin responses and reduce the risk of type II diabetes, cardiovascular disease and obesity [48]. The mortality caused by diabetes increased by 80% in the past twenty years, and it is projected to increase to over 6 million people by 2040 [49]. Thus, it's important to generate high RS and low GI rice as a better source of starch for diabetes patients.

SBE is a key enzyme in starch biosynthesis, forming the branched structure of amylopectin by catalysis of glucose monomer binding through α-1,6 bonds [50]. *SBEIIb* deficient mutants and transgenic rice have been shown to accumulate greatly modified starch with higher RS and amylose contents and higher onset temperature for gelatinization. *SBEIIa* and *SBEIIb* usually produce short chains while *SBEI* usually produces long chains of amylopectin . Down-regulating or eliminating the expression of *SBEI*I in several major crops also leads to increased contents of RS and AC. For example, editing the exon 12 or exon 18 of Os*SBEIIb* in the *Japonica rice* cultivar Nipponbare through CRISPR/Cas9 significantly decreases its expression level and significantly increases the contents of RS and AC in the endosperm. In the present study, we developed seven Os*SBEIIb* 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. Both knock-out and knock-down of *OsSBEIIb* lead to decreased gene expression, *OsSBEIIb* enzyme activity 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 As expected, *OsSBEIIb* expression in the homozygous mutants is almost completely knocked down since rice genome contains only one copy of the gene. The transcript of *OsSBEI* was unaffected in *OsSBEIIb* RNAi mutant rice seed. Consistently, the mRNA level of *SBE*I is not affected at all in the mutant lines obtained in the current work. Following the suppression of *OsSBEIIb* expression, the total *SBE* activities in both heterozygous and homozygous lines are significantly reduced. The *SBE* activity remaining in the mutant lines is presumably due to the normal expression of other *SBE*s, as reported previously [51-59].

In wheat, RNAi inhibition of *TaSBEIIa* and *TaSBEIIa* at the same times leads to increased RS from 1.6% to more than 35% and AC from 25.5% to more than 80% [60]. Amylose-only barley with very high RS content (65%) is generated when the three Hv*SBE* genes are simultaneously suppressed by RNAi [61]. The RS content of ordinary maize starch is as low as 1%-2% and its AC is about 30% [62]. *ZmSBEIIb* is the predominant isoform in the maize endosperm which is at least 50 times the abundance of *ZmSBEIIa*. The RS and apparent AC are as high as 34.3% and 85.6%, respectively, in the Amylose Extender (AE) mutant maize which lacks Starch Branching Enzyme *IIb* (*SBEIIb*) activity. *OsSBEIIb* is also the predominant *SBE*II isoform in rice endosperm, and chemical mutagenesis of *OsSBEIIb* in the *Japonica rice* cultivar Jiangtangdao 1 significantly increases RS content from 0.4% to 11.7% and the AC from 16.2% to 31.1% suppression of *OsSBEIIb* expression mediated by artificial microRNA (ami-RNA) in the *Japonica* cultivar Nipponbare also significantly increases RS from 0.1% to 4.4% and the AC from 19.6% to 41.2% while GI decreases from 85 to 44 Targeted editing of *OsSBEIIb* by CRISPR/Cas9 in the *Japonica* cultivar Kitaake increases its RS from 0.1% to 6.5% and AC from 15% to 25% with a 30% reduction in grain weight 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, which is negatively correlated with the expression of *OsSBEIIb*. The averaged AC content of *OsSBEIIb* homozygous mutant endosperms is 2-fold higher (31.8%) than that of WT (15.8%) and 1.2-fold higher than that of *indica* variety TNGS14 (26.8%). The corresponding value for the heterozygous mutant lines (23.4% AC) is 1.48 fold higher than that of WT, respectively. These results are consistent with previous studies in rice Satoh et al showed that Os*SBEIIb* 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 The degree of chalkiness of our mutant grains is similar to that of *OsSBEIIb* mutant rice obtained in the previous study.

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. The RS and AC in rice starch are negatively correlated with the rate of digestibility (i.e., release of reducing sugar during digestion) and GI. The higher the AC in the grain, the lower the digestion efficiency of starch because the hydrogen bonds that exist in the glucose chain connecting amylose molecules make it more resistant to α-amylase. The digestibility, as indicated by the 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, due to increased RS and AC. 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. This is related to the higher proportion of RS, long-chain amylopectin, AC and high semi-crystalline 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. 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 of 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).

Sun, et al. [22] 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 the homozygous mutant lines with long fragment insertion (Line-34, 60) and short fragment insertion (Line-33, 42,43) as well, 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 of 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 which may not affect the ability of the mutated *OsSBEIIb* to function. 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 AC 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. The AC in rice is negatively correlated with Breakdown Viscosity (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. 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. The high helix conformation of amylose with more hydrogen bonds requires a higher temperature to gelatinize, leading to an increase in gelatinization temperature. 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.

In this study, we demonstrate the advantages of CRISPR/Cas9-mediated genome editing technology in breeding high-RS 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. Moreover, the transgenes used for editing can be easily eliminated from the segregating progenies. Also, besides *OsSBEIIb* the expression of other related genes, such as *OsSSIIIa*, *OsSBEI* and *OsSBEIIa*, can be down-regulated while the expression of *OsW$_x$III* can be overexpressed at the same time through CRISP/Cas9 to provide more suitable sources of starch for type II diabetes.

CONCLUSION

In the present study, high RS and AC rice with a significantly lower GI was efficiently 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. Similarly, the level of gene expression and enzymic activity of *OsSBEIIb* were inhibited more significantly in the homozygous than heterozygous mutant lines while the RS contents in the homozygous and heterozygous mutant lines were 25 and 15.6-fold higher than those in WT (TNG82), and the AC in the homozygous and heterozygous mutant lines were 2.0 and 1.48 fold higher than that in WT (TNG82), respectively. With increasing RS and AC, the GI was also progressively decreased in heterozygous and homozygous mutant endosperms. Moreover, the transgene-free plants can be readily screened from T1 generations. In summary, these results demonstrate the 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.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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