Original Research Article

Biochemical analysis of *SBEIIb* mutant rice lines generated through CRISPR/Cas-mediated targeted mutagenesis

Abstract

Knock out of the Starch branching Enzyme [SBE] encoding genes lead to increase in the Apparent Amylose Content in cereals. Reduced SBE activity decreases the frequency of branch points in the Amylopectin fraction, while increasing the Amylose Content [AC]. AC is positively correlated to the Resistant Starch [RS], which acts as dietary fibre leading to lowering Glycemic Index [GI] . Among the 4 Starch branching enzyme encoding genes, *SBEIIb* is exclusively expressed in Rice Endosperm. Therefore, in this study, the homozygous *OsSBEIIb*mutant lines of CO51 rice cultivar generated through CRISPR/cas9 were selected and biochemical analysis was carried out to determine theAC. Among the events studied, all the eventsshowed a relatively increased AC as compared to the wild type. These findings highlight the need of identifying and developing rice genotypes with high RS and Amylose, which can be suitable for consumption by people suffering from diabetes, obesity, and other colon related illnesses.

Key Words: Rice, Starch branching enzyme [SBE], Amylose , Biochemical analysis, CRISPR/Cas9.

Introduction:

Globally, the prevalence of chronic diabetes mellitus is rising quickly [1]. Diabetes often manifests clinically as high blood sugar levels brought on by a total lack of insulin or insulin resistance. Long-term, uncontrolled insulin deficiency and high blood glucose levels severely compromise the functioning of numerous organs and lead to a host of serious problems, including heart disease, kidney disease, nerve damage, and eye illness [1].

For about half of the world's population, rice is a significant cereal crop [2; 3]. The major composition of a rice grain is made up of starch, which is an essential source of energy for people [4; 5]. Linear amylose, in which the monosaccharide units are primarily linked by α-1,4 -glycoside bonds, and highly branched amylopectin, which contains extra α-1,6-glycoside branches, are two types of starch that may be distinguished based on the degree of branching and the shape of the glycosidic linkages [4; 5; 6]. In 1982, in addition to the conventional starch classifications, the idea of resistant starch (RS) was proposed [7]. RS is a kind of starch that is resistant to amylase hydrolysis and is rarely digestible in the human small intestine [5;8]. Instead, colonic bacteria digest it in the large bowel to form short-chain fatty acids (SCFAs)[8; 9]. However, most rice varieties have a low RS content [5; 2; 3].

The high-RS diet has potential benefits for the overall gut health [8; 9; 10; 11]. In particular, a diet with a high proportion of RS is beneficial for diabetic patients in reducing the postprandial blood glucose levels and alleviates insulin resistance due to its slow digestion in the small intestine [8; 11]. RS content is favorably connected to AAC, including amylose, amylopectin long-branch chains, and intermediate components in cereals [11; 12; 13].

Starch biosynthesis is a complex mechanism composed of four enzymes such as, ADP-glucose pyrophosphorylase, starch synthase, starch branching enzyme (SBE), and starch debranching enzyme. Each of these enzymes is made up of many subunits and has different isoforms. Each enzyme has a different purpose, although they are most likely part of a network. Genes governing amylose synthesis also influence amylopectin formation, and amylopectin can give rise to amylose [14]. Increasing the amylose content *via* increasing the expression of the GBSSI enzyme typically requires gain-of-function mutations that are difficult to introduce. As a result, directly raising the amylose content to develop transgene-free germplasm with high RS content is inefficient. It has been demonstrated that increasing AAC by regulating the production of amylopectin is a simpler method [10; 15; 14].

SBEs are important in amylopectin biosynthesis because they catalyse chain transfer by cleaving α-1,4 linkage after condensation of α-1,6 linkage. SBEs are classified into four classes in cereals such as rice, maize, barley, and wheat: *SBEI*, *SBEIIa*, *SBEIIb* and *SBEIII* [16]. *SBEI* is involved in the synthesis of long chains of amylopectin in rice, *SBEIIb* is involved in the formation of short chains, whereas *SBEIIa*has minor role in formation of short chains [17]. Cereals have been genetically

modified to boost AC by inhibiting amylopectin production by mutation or suppression of *SBE* gene expression [10].

Previous research has found that *SBEIIb*, which is mostly found in endosperm, has a higher impact on starch composition than other isoforms [15; 17; 18]. Regardless of genetic background, the AAC was nearly doubled in sbeIIb mutants created by chemical mutagenesis [18; 19; 20]. As mutagenesis is unpredictable, crop breeding with chemical or physical mutagens usually necessitates arduous and time-consuming processes to test mutants of a specific gene [15; 21; 22]. CRISPR/Cas9-mediated mutagenesis can circumvent the constrains of random mutagenesis and RNAi by producing site-specific changes directly through exact complementary base pairing [23; 24].

In this investigation, we analysed the starch content of mature grains of the transgenic lines and their wild-type progenitors to study the effect of targeted mutagenesis of *SBEIIb* on the amylose content of rice grain.

Materials and methods

Plant material:

The *SBEIIb* mutants generated earlier in our lab. A total of thirteen T_1 lines were raised in transgenic greenhouse under the hydroponics system. The T_1 lines were Sanger sequenced for the selection of homozygous line. Out of thirteen events, three homozygous events were identified and their T_2 seeds were harvested. For the biochemical analysis, the T_2 seeds were taken with three biological replicates and three technical replicates .

Biochemical analysis

Starch and amylose content estimation for the three homozygous events were carried out. The steps involved in the starch and amylose content estimation are detailed below.

Starch estimation

The estimation of starch was carried out by using Anthrone reagent. The samples (Seeds) were homogenized in a pestle and mortar. The sample (0.1 to 0.5 g)

was taken and mixed with 50 ml of hot 80 % ethanol. Then the mixture was centrifuged and the pellet was washed twice using hot 80 % ethanol, then dried thoroughly over a water bath before cooling it in ice/normal water. After drying, 5 ml of water and 6.5 ml of 52 % perchloric acid was added to the pellet in each tube. The tubes were then incubated on ice for 20 minutes and centrifuged at 10,000 rpm for (8-10 minutes). The above extraction with fresh perchloric acid was done for twice. Then the supernatants were pooled together in a single falcon tube. From that 0.2 ml of the supernatant was taken and the final volume was made 1 ml of distilled water in 2 ml Eppendorf tube. This solution serves as the working standard for further analysis.

The starch standards were prepared by putting 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, and 1 ml of supernatants in each tube and filling to 1 mL with water. Next each tube was filled with 4 ml of Anthrone reagent. After cooling it in ice water, intensity of green to dark green colour at 630 nm was measured by spectrophotometer.

Apparent amylose content estimation

Apparent AC was determined following a modified method according to the iodine adsorption method of Konik-Rose *et al*[25]. The experiments were performed in triplicate. Seeds were weighed and crushed to get a sample volume of 50 mg and added to 50 ml volumetric flask. To this,0.5 ml of 95 % ethanol was added to wash down the sample adhering to the flask followed by 5 ml of 1 N NaOH. The above mixture was incubated for 15 minutes in a water bath to gelatinize the starch by boiling. Stock was prepared by taking the 4 ml of boiled extract and added to new flask to make up the volume to 50 ml. From this, 0.5 ml was pipetted out to a test tube and 2.0 ml water was added to make up the volume to 2.5 ml. To this, a drop of phenolphthalein was added to develop pink colour. 0.1 N HCI was added drop by drop until it turn to colourless. Thevolume was made up to 10 ml with distilled water after addition of 1 ml of iodine reagent and the blue colour developed was read at 630 nm. Amylose concentration was obtained by plotting the absorbance in the standards curve. AC of each sample was expressed as percentage to total quantity of sample taken for analysis. The amylopectin concentration was calculated by subtracting the amylose percentage from the total starch percentage of the sample.

Statistical analysis of the biochemical observations

Data on the starch and amylose from 9 replicates (3 biological replicate and 3 technical replicate) of each event were expressed as mean ± standard deviation. The

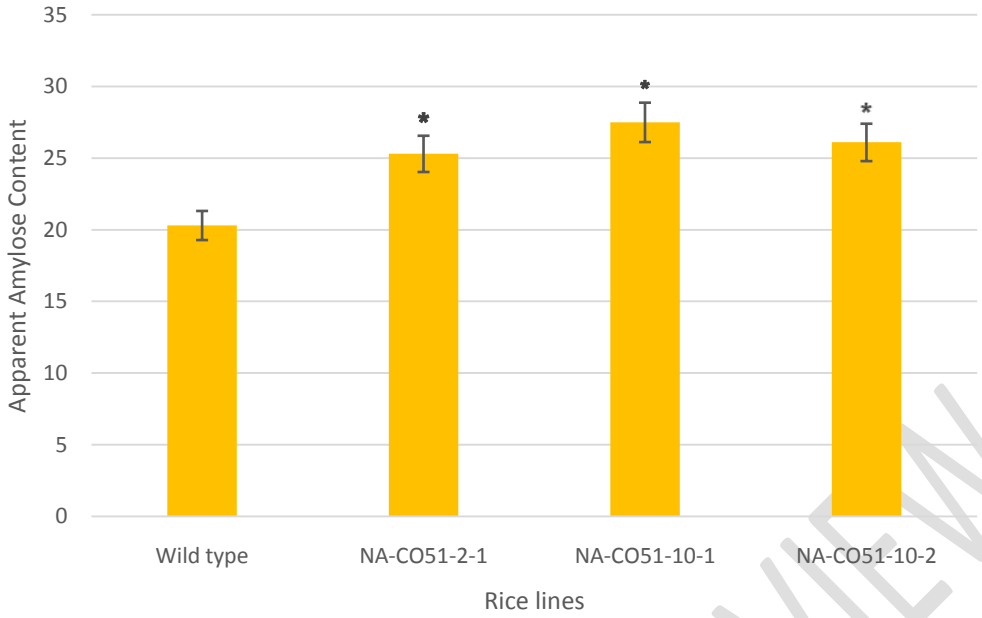
data was subjected to one way analysis of variance (ANOVA) to calculate the critical difference at 5 % level of significance. Mean values of Starch and Amylose for each event was compared with that of wild type CO51 using student's t-test to find the presence of any significant difference between the mutants and the wild type.

Results and Discussion

Plant mutants are useful genetic materials for identifying gene function and regulation, as well as providing germplasm for crop improvement. Physical mutation, chemical mutation, and T-DNA insertion mutation are examples of traditional mutation processes. However, their mutation ratios are modest, and the mutation sites are random and unknown. The CRISPR/Cas9 system is capable of rapidly producing target gene mutations and has been widely used in the generation of plant mutants, particularly in reverse genetics for exposing target gene function and control [26;27; 28; 29; 30].

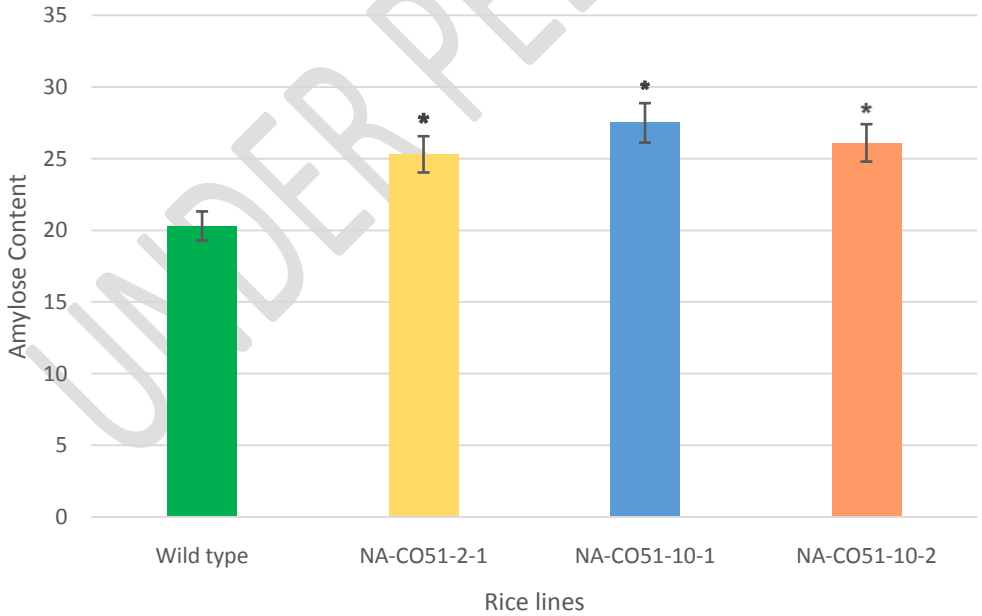
The total endospermic starch content of rice is typically composed of a 0-30 % amylose fraction and a 70-100 % amylopectin fraction [31]. However, rice types can be classified as higher([25-33 %), intermediate (20- 25%), low (12-20 %), very low (2-12 %), or waxy (0-5 %) based on their amylose concentration [32].

The chosen three homozygous events (NA-CO51-2-1,NA-CO51-10-1, NA-CO51-10-2) were analysed for the Starch and Amylose content. The ACs of wild type were 20.3 %. Both AAC and AC increased significantly($p<0.05$) in the three events, with AAC ranging from 25-27 % in the three homozygous mutant lines (Fig.1). On average, the AC increased from 20.3 % in the wild type to 27.5 % (1.35 fold increase) in the homozygous mutant lines (Fig. 2). The increased AC in the grains of these mutant lines did not affect the total starch content; the total starch contents in the mutant lines were similar to that in wild type (Fig. 3). As expected, the ratio of amylose/amylopectin increased significantly in the mutant lines, relative to wild type (Fig. 4). Taken together, these results clearly show that a knockout of *OsSBEIIb* in rice can increase AC from 20.1 % to 25–27 %. Clearly, suppression of *OsSBEIIb* expression for amylopectin biosynthesis in rice leads to increased AC.



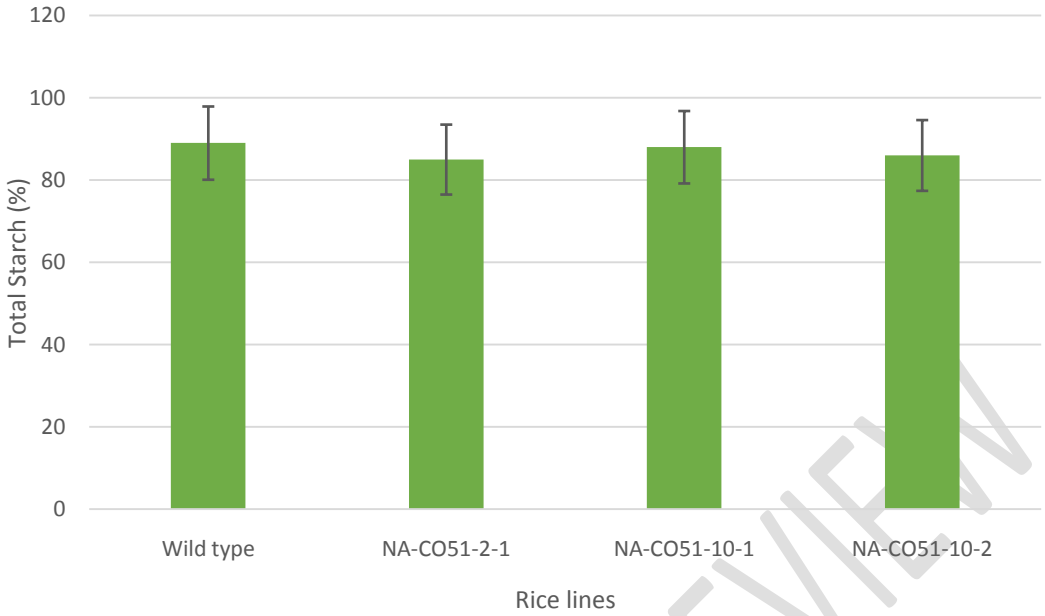
*Significant differences between mutant and wild-type lines [t-test, P < 0.05].
Mean ± SD is calculated from duplicate measurements. Values with different letters in the same column are significantly different with p < 0.05

Fig.1.Apparent Amylose Content [%]



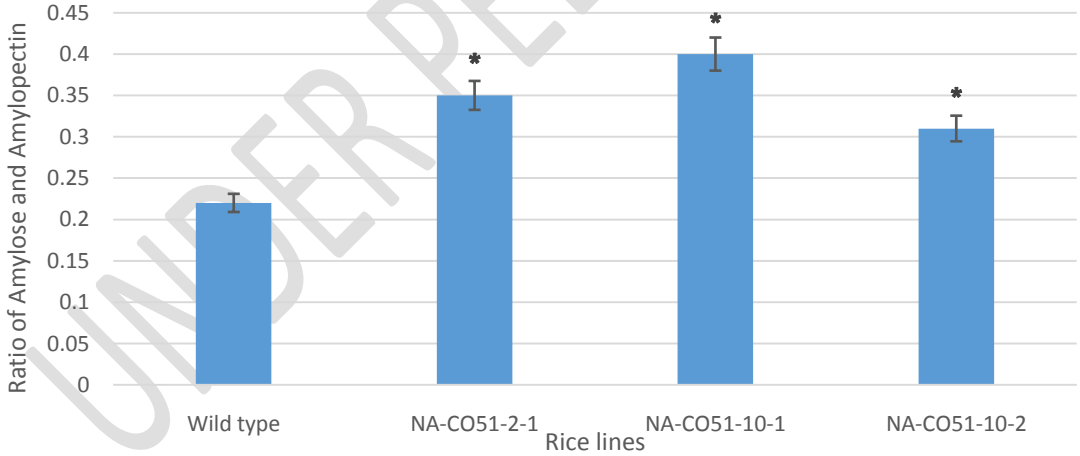
*Significant differences between mutant and wild-type lines [t-test, P < 0.05].
Mean ± SD is calculated from duplicate measurements. Values with different letters in the same column are significantly different with p < 0.05

Fig.2.Amylose content [%]



*Significant differences between mutant and wild-type lines [t-test, P < 0.05].
Mean ± SD is calculated from duplicate measurements. Values with different letters in the same column are significantly different with p < 0.05

Fig.3. Total Starch [%]



*Significant differences between mutant and wild-type lines [t-test, P < 0.05].
Mean ± SD is calculated from duplicate measurements. Values with different letters in the same column are significantly different with p < 0.05

Fig.4. Ratio of Amylose/Amylopectin

Previous research on the *indica* rice cultivar (TNGS14) observed that AC increased dramatically from 15.8 % in wild type to 24.1 % in the heterozygous

condition and 30.8 % in the homozygous mutant lines [33]. Baysal *et al*. [34] developed and analyzed T_3 seeds from a homozygous mutant (E15 CRISPR/Cas9 edited line) with a 4 bp deletion, observed a 1.4 fold increase in amylose content in the edited line (27.4 %) compared to the wild type *japonica* cultivar Nipponbare (19.6 %). Sun *et al*. [15] demonstrated a considerable increase in the proportion of amylose (25 %) and resistant starch (5-8 %) in *OsSBEIIb*mutant lines of cultivar Kinmaze. The amylose/amylopectin fraction was also analysed and found to vary in the edited homozygous lines when compared to the wild type CO51.

Furthermore, for enhancing GBSS, targeting all the four SBEs or a combination of inhibiting soluble starch synthesis and enhancing GBSS, may lead to a very high-amylose starch in rice.

Conclusion

In light of the results of the present investigation, it can be concluded that three homozygous mutant lines generated through CRISPR/Cas9-mediated targeted mutagenesis has shown a considerable increase in the amylose content as compared to the wild type. Therefore it gives a clear demonstration of the amylose content being increased due to the indel mutations created in the rice *SBEIIb* gene.

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