RNA interference-mediated silencing of the starch branching enzyme gene improves amylose content in rice

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ABSTRACT. Amylose and amylopectin are the 2 major components of plant storage starch. The rice starch branching enzyme (RBE) plays an important role in the starch components of rice. In the present study, we selected a specific 195-bp segment from the *RBE3* gene to construct hairpin DNA, which was driven by an endosperm-specific high molecular weight glutenin promoter to regulate the biosynthesis of starch. An RNA interference plasmid for the *RBE3* gene was constructed to form double-stranded RNA. Following *Agrobacterium*-mediated rice transformation (in the cultivar Zhonghua 11), 41 transgenic plants were identified using PCR and Southern blot analysis. Semi-quantitative real-time PCR revealed that *RBE3* gene expression was significantly reduced in immature transgenic seeds. Transgenic rice amylose content had an average increase of 140%. The highest rice amylose content was 47.61% and the growth rate increased 238% compared to the non-transgenic controls. Branching enzyme II activity was notably reduced, and ADP-glucose pyrophosphorylase, soluble starch synthase,

isoamylase, and pullulanase enzyme activity was markedly reduced in T_3 seeds. Relative enzyme activity change explained the reduction in thousand-grain weight in transgenic plants. The present study indicated that amylose content was negatively correlated with branching enzyme II activity, spike size, and thousand-grain weight.

Key words: Rice; *RBE*3 gene; RNA interference; Amylose content

INTRODUCTION

Starch is the main stored carbohydrate in plants and is the primary component of food crops (Kuipers et al., 1994). Amylose and amylopectin are the 2 components of starch. Amylose is essentially a linear molecule in which glucose residues are joined via an α-(1,4) glucosidic bond. Amylopectin consists of highly branched glucan chains, including chains of roughly 20 α-(1,4)-linked glucose residues joined by α-(1,6) linkages to other branches (Mukerjea and Robyt, 2010). The proportion of amylose to amylopectin is a key factor in determining starch quality. Low amylose and high amylopectin levels are largely applied in food production; high amylose and low amylopectin levels are widely applied in industries (Liebl et al., 1997; Desai, 2007). Therefore, research aimed at regulating starch biosynthesis and improving starch quality is imperative. Developments in molecular biology have resulted in various starch biosynthesis management methods, ranging from genetic engineering to conventional breeding (Hirose and Terao, 2004; Song et al., 2004; Leterrier et al., 2008).

Starch is manufactured via the activity of starch synthesizing enzymes during a long developmental period (Fontaine et al., 1993). Starch biosynthesis involves the activities of 4 enzymes, that is, ADP-glucose pyrophosphorylase (ADPGPPase), starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE; Ball et al., 1996); these enzymes catalyze the shape of ADP-glucose, prolong glucan chains, form branching chains, and mediate starch degradation, respectively. Previous research has demonstrated that amylose is directly catalyzed by granule-bound starch synthase (GBSS or WAXY). Furthermore, amylopectin is synthesized by the production of a joint reaction between SBE, soluble starch synthase (SSS), and DBE; therefore, rice starching branching enzyme 3 (RBE3) is considered critical in amylopectin synthesis. Previous studies indicate that the strategy for improving starch quality should focus on regulating levels of specific enzymes or on harmonizing key enzymes (Hirose and Terao, 2004; Chen et al., 2006; Zhang et al., 2007).

Previous research has shown that RNA interference (RNAi) is an approach that effectively controls gene expression. To date, RNAi has been extensively employed to inhibit gene expression in plants, epiphytes, nematodes, protozoa, hexapods, and mammals (Mao et al., 2007; Jain et al., 2008a; Kim et al., 2009; McGinnis, 2009). In the present study, we constructed an *RBE3* gene siRNA expression plasmid for efficient regulation of rice starch, which contained an *RBE3* gene segment inverted repeat structure. The rice was transformed with an RNAi vector, resulting in *RBE3* gene expression degradation of the endogenous homologous gene, thereby obtaining an *RBE3*-deficient mutation. These results provide basic data for *RBE3* function as related to starch structure and quality, and its regulation network for crop seed characteristics.

MATERIAL AND METHODS

PCR amplification of the *RBE3* gene segment

Rice (*Oryza sativa* sp *japonica*) variety Zhonghua 11 young leaves and immature seeds were collected at the seedling and filling stage for DNA and RNA isolation. The nucleotide sequence of the *RBE3* gene was verified according to the principles of siRNA design. The segment was located within 66,197 and 66,391 bp of the rice genome sequence, for a total of 195 bp. The sense primer: RBE3i-F, 5'-GCGGATCCGGAAGTAGCGATTAACGTGTT-3', and the antisense primer: RBE3i-R, 5'-GCGTCGACTAGCTTTACCTTTGCCCCTT-3', were used. A 50-μL reaction comprising the following products was used: 1 μL 10 mM dNTP, 1 μL 1 μg/μL DNA template, 3 μL 10 μM primers, 5 μL 10X PFU buffer, and 1 μL 5 U/μL PFU DNA polymerase, followed by addition of MilliQ H_2O to reach the final volume. PCR amplification conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 45 s, extension at 72°C for 30 s; followed by a final extension at 72°C for 8 min.

Construction of siRNA expression plasmid and transformation

The constructed siRNA expression plasmid included a 195-bp inverted repeat spliced by a gibberellin 20 (GA20)-oxidase intron 1 fragment driven by an endosperm-specific, high molecular weight glutenin (Glu) promoter. The gene expressing the siRNA of the *RBE3* gene was transferred to the pCAMBIA1301 vector, which contained the kanamycin gene as a selection marker. This plasmid was designated pRBEi.

In the T-DNA of p1300 (RBE3i), the inverted repeat was spliced by GA20-oxidase intron 1 and driven by an endosperm-specific, high molecular weight Glu, Glu-1D-1 gene promoter, using the *bar* gene as the selectable marker. The binary vector was transferred to *Agrobacterium* strain AGL0. The rice transformation system was utilized according to methods described elsewhere (Hiei et al., 1994; Yookongkaew et al., 2007).

Molecular characterization of transgenic plants

Genomic DNA of T_0 transgenic plants was extracted from young leaves using the cetyltrimethylammonium bromide method. The specific primers, 5'-ATGAGCCCAGAAGACG-3' and 5'-TCAGATCTCGGTGACGG-3', were used for amplification of the *bar* gene. PCR amplification conditions were as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s; followed by a final extension at 72°C for 8 min. Transgenic plants were positively identified by PCR and were further characterized using Southern blot analysis. The *bar* gene (550 bp) was digested from the pCAMBIA1300 plasmid and labeled with digoxigenin as a Southern blot probe. A digoxigenin High Prime DNA Labeling and Detection Starter Kit (Roche, Mannheim, Germany) was used for Southern blotting.

To examine the expression of the *RBE3* gene by semi-quantitative real-time PCR, total RNA from untransformed and transgenic plants was isolated from immature seeds. The specific primers actin (Act)-F, Act-R, RBE3-F, and RBE3-R were synthesized according

to the rice actin and *RBE3* genes. A First-Strand cDNA Synthesis Kit (Shenergy Biocolor Company, Shanghai, China) was used for reverse transcription experiments. The actin gene was used as the internal control. PCR amplification conditions were as follows: initial denaturation at 95°C for 2 min; followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s; followed by a final extension at 72°C for 8 min. The primer sequences were as follows: Act-F: 5'-CCCTTGTGTGTGACAATGGAACT-3', Act-R: 5'-GACACGGAGCTCGTTGTAGAAGG-3', RBE3-F: 5'-ATGAGTTCGGACATCCTGAAT GG-3', RBE3-R: 5'-CATTCCGCTGGAGCATAGACAAC-3'.

Amylose content measurement

The T_1 seeds were cut with clean blades, and embryos were cultured in Murashige and Skoog medium. Seedlings were cultivated on plastic plates to reproduce T_2 seeds. Homozygotes were selected by continued PPT addition. Endosperm was selected to measure amylase content by iodine colorimetry in homozygote plants.

Enzyme activity and agronomic characteristic analysis of transgenic lines

Following 15 days of flowering, rice grains were randomly chosen in each transgenic line for endosperm analysis. Branch enzyme II (BEII) activity was analyzed according to a previously described method (Mutisya et al., 2009). Ten plants were randomly chosen from each T_3 generation homozygote to analyze plant height, effective number of grains per spike, thousand-grain weight (TGW), and amylose content.

Further TGW reduction was assessed in the T_3 generation of C18, C16, and Zhonghua 11 transgenic lines to detect starch synthesis activity relative to the following enzymes: ADPGPPase enzyme activity was analyzed according to the method described by Siedlecka et al. (2003), SSS and GBSS were evaluated according to the method of Shimonaga et al. (2007), and isoamylase and pullulanase activity was assessed by the method described by Kubo et al. (1999).

RESULTS

Construction of expression vector and molecular identification of transgenic rice

The siRNA expression vector T-DNA structure is shown in Figure 1. The PCR results indicated that 41 of 64 individual plants were positive transgenic plants, and the positive ratio was 64.6% in all plants. All T_0 transgenic lines were analyzed by Southern blot probed with the *bar* gene. In all 6 transgenic lines, 1 single-copy transgenic event was universal, while tandem insertion of multiple copies did not occur in transgenic rice (Figure 2). Untransformed plants exhibited the absence of a band, indicating that hybridization was specific to transgenic plants.

Expression of the *RBE3* gene and amylose content determination

Semi-quantitative PCR was used to determine whether *RBE3* mRNA expression was reduced following induction in transgenic immature rice seeds (Figure 3). The results revealed a notable reduction in *RBE3* gene expression in all T_0 transgenic lines. This indicated that the

constructed dsRNA effectively inhibited *RBE3* gene expression. The actin gene transcripts remained at expected levels in all of the lines.
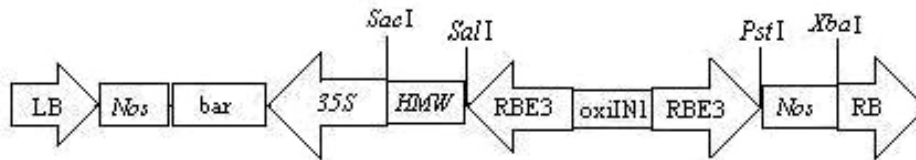

Figure 1. T-DNA structure in the pRBEi siRNA expression vector. The inverted repeat was spliced by GA20-oxidase intron 1 and driven by an endosperm-specific, high molecular weight glutenin promoter, using *kan* as the transgenic plant selectable marker.
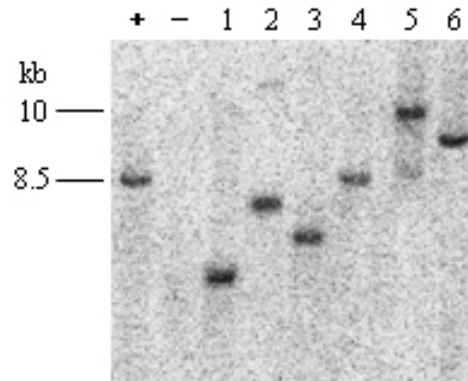

Figure 2. Transgenic plant Southern blot analysis. *Lanes 1* to *6* = Southern blot analysis of 6 transgenic plants using the *bar* sequence as a probe, indicating a copy insertion; *lane* (-) = negative control, DNA from non-transformed Zhonghua 11; *lane* (+) = positive control using plasmid DNA pRBEi as a control.
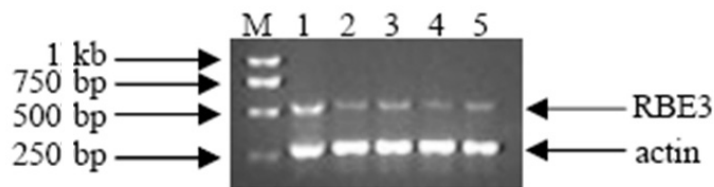

Figure 3. Semi-quantitative real-time (RT)-PCR analysis of RBE3 in T_1. *Lane M* = DNA marker DL 2000; *lane 1* = positive control, PCR using reverse-transcribed cDNA from untransformed Zhonghua 11, an expected 510-bp RBE3 fragment was clearly evident; *lanes 2* to *5* = semi-quantitative RT-PCR analysis of 4 transgenic plants, the expected 510-bp RBE3 fragment was evident; internal control was a 270-bp actin fragment.

In addition, 20 of the 41 transgenic lines were randomly selected for amylose content measurements in brown rice. Corresponding amylose content was calculated using the regression equation (standard curve and regression equation are not listed). The results showed that the difference between transformed and untransformed seed amylose content was significant

(P < 0.01; Table 1). The performance of amylose content in all 20 transgenic lines was significantly increased by an average of 140%. Amylose content increased from 14.09% in untransformed rice to 33.86% in transformed rice. Amylose content in the C18 line reached 47.61%, indicating a 238% increase.

Table 1. Amylose content from different lines of T_1 seeds.

Line	Amylose content (%)[a]	Line	Amylose content (%)[a]
C01	32.27 ± 0.19	C26	32.73 ± 0.26
C02	39.43 ± 0.19	C27	22.27 ± 0.27
C03	41.93 ± 0.23	C30	30.80 ± 0.21
C07	34.77 ± 0.17	C32	31.70 ± 0.24
C10	24.43 ± 0.22	C33	33.86 ± 0.10
C12	37.05 ± 0.17	C37	30.00 ± 0.23
C16	31.82 ± 0.19	C38	28.18 ± 0.07
C18	47.61 ± 0.29	C39	40.00 ± 0.13
C19	41.59 ± 0.30	C41	35.00 ± 0.29
C23	29.66 ± 0.26	C25	32.16 ± 0.23
Average of transgenic seeds	33.86 ± 1.36	Zhonghua11	14.09 ± 0.09

Data are reported as means ± standard error. [a]Difference between amylose content in transgenic and untransformed seeds was significant (*t*-test, P < 0.01).

Seed and spike traits in T_1 transgenic rice

Transgenic rice traits were investigated by assessing T_1 seed and spike attributes (Figure 4). Transgenic and control rice exhibited different traits. Seeds of T_1 transgenic rice were whitish and friable compared to untransformed seeds. Furthermore, transgenic rice seeds were opaque and wrinkled, while untransformed seeds were full. The transgenic rice spike was thin and shriveled compared to the untransformed rice spike.

Figure 4. Transgenic traits. **A.** Rice spikes. Control 1 depicts a robust and full untransformed rice spike; 2-4 represent thin and shriveled transgenic spikes. **B.** Seeds. Control 1 displays an untransformed large and plump seed; 2-5 indicate small and shriveled transgenic seeds. **C.** Seed transparency. Control 1 represents a bright seed; 2-5 display opaque seeds.

Agronomic traits and enzyme activities in T$_3$ transgenic rice

Characterization of Zhonghua 11 and T$_3$ transgenic rice, including amylose content, plant height, effective grain number, and TGW were investigated in detail (Table 2). Results showed that BEII activity, effective number of grains per spike, and TGW were markedly reduced in T$_3$ transgenic rice. In addition, transgenic rice had high amylose content relative to that of Zhonghua 11. Other key enzyme activities involved in grain starch synthesis were assessed in the T$_3$ generation C0322 and C3931 transgenic lines. The results showed an obvious decrease in BEII activity (Table 3). The BEII activity in the C0322 transgenic line decreased by 71.1%, with differences observed between lines. Furthermore, gene silencing also led to a significant reduction in ADPGPPase, SSS, and DBE activity, with a slight increase in GBSS activity.

Table 2. Trait determination in T$_3$ transgenic rice.

Lines	Proportion (%) amylose content	Plant height (cm)	Effective grain number	Thousand-grain weight (g)
Zhonghua 11	14.42 ± 0.19	92.46 ± 0.13	136.1 ± 6.9	32.84 ± 0.31
C0221	33.33 ± 0.31	77.96 ± 0.42	98.4 ± 5.8	30.12 ± 0.13
C0322	38.43 ± 0.24	81.21 ± 0.48	84.2 ± 8.2	28.21 ± 0.21
C1212	34.15 ± 0.91	88.35 ± 0.36	93.7 ± 8.4	29.43 ± 0.19
C3321	31.47 ± 0.71	86.52 ± 0.43	94.2 ± 7.7	30.45 ± 0.33
C3931	30.16 ± 0.32	86.46 ± 0.51	113.3 ± 6.7	29.67 ± 0.27
C4143	22.31 ± 0.19	90.28 ± 0.21	118.5 ± 7.6	30.67 ± 0.25

Table 3. Enzyme activity determination in T$_3$ transgenic rice.

Enzyme types	Zhonghua 11	Transgenic line C0322	Transgenic line C3931
BEII	32.2 ± 0.76	9.3 ± 0.43	13.7 ± 0.52
ADPGPPase	125.1 ± 3.51	107.6 ± 3.09	113.3 ± 3.31
GBSS	15.6 ± 0.32	16.5 ± 0.71	16.1 ± 0.65
SSS	55.7 ± 1.31	47.5 ± 1.32	51.7 ± 1.39
Pullulanase	42.5 ± 1.12	37.9 ± 1.02	39.1 ± 1.09
Isoamylase	19.5 ± 0.72	17.2 ± 0.71	17.9 ± 0.59

BEII = branching enzyme II; ADPGPPase = ADP-glucose pyrophosphorylase; GBSS = granule-bound starch synthase; SSS = soluble starch synthase.

DISCUSSION

Starch is an important contributing factor to grain yield and rice quality. In recent years, cloning of starch enzyme genes has improved our understanding of starch synthesis (Jain et al., 2008b; Leterrier et al., 2008). Several cDNAs encoding SBEs have been isolated from rice, wheat, maize, and barley (Kim et al., 1998; Mutisya et al., 2003; Han et al., 2007). Four classes of enzymes catalyze starch biosynthesis, that is, AGPase, SS, BE, and DBE. For example, SS elongates glucans by adding Glc residues from ADP-Glc to the glucan nonreducing ends through α-1,4 linkages. The *WAXY* gene, located on rice chromosome 6, encodes the GBSS enzyme, which plays a key role in amylose synthesis (Chen et al., 2006). BEII proteins play a large and specific role in amylopectin synthesis.

The development of molecular biology techniques makes it possible to regulate the activity of key enzymes in starch biosynthesis and modify the content and structure of starch by genetic engineering. Many plant genetic transformation systems have been successively applied, thereby

improving starch quality (Ryoo et al., 2007). Excessive expression of starch synthesis-related genes, including expression of endogenous genes and foreign genes, has been studied in recent years (Toyota et al., 2006). The *AGPP* gene glgC16 (regulation of variable configuration is not sensitive) from mutant strains of *Escherichia coli* 618 was used to transfer a tuber-specific expressed gene promoter into potatoes. Tuber starch content increased an average of 35% in the transgenic potatoes.

Furthermore, silencing of endogenous genes by antisense RNA or RNAi technology has also resulted in significant progress. Antisense RNA technology was employed to silence expression of the *GBSS* gene in potatoes, resulting in a decrease in GBSS enzyme activity, which led to a sharp decline in potato tuber amylose content (down from 70 to 100%; Kuipers et al., 1995). Similar use of antisense RNA technology in cassava, rice, and other plants also resulted in transgenic plants with improved starch content. In this study, RNAi technology was employed to silence *RBE3* gene expression, and the endosperm-specific promoter Glu was selected to control its expression. siRNA structure played a specific interference role in transgenic rice, resulting in marked improvement of transgenic rice seed amylose content, with an average increase of 140%. These observations indicated that the function of *RBE3* is dominant to amylose content in rice endosperm. Expression of *RBE3* was negatively correlated with amylose content.

T_3 transgenic rice was further evaluated to determine trait and amylose content differences relative to controls. Amylose content of T_3 transgenic rice was stable and exhibited higher levels than that of the control. Furthermore, seeds of transgenic rice were smaller than untransformed seeds, the endosperm structure was loose, and the TGW was lower than that of untransformed seeds. This may be due to the fact that the primary rice starch component is amylopectin and that the *RBE3* gene plays a leading role in amylopectin formation. Therefore, a reduction in activity caused a direct decrease in TGW. The enzyme activity of the T_3 generation showed that *RBE3* RNAi not only resulted in a decrease in *RBE3*, but amylopectin synthesis was inhibited and amylose content significantly increased. In addition, it also led to a decrease in the activity of other key starch synthesis enzymes, i.e., ADPGPPase, SSS, and DBE were significantly reduced in transgenic seeds. Consequently, endosperm synthesis was disturbed by reduced enzyme activities. This is a viable explanation for the decline of TGW in transgenic rice. Taken together, the results of the present study suggest that the *RBE3* gene plays a critical role in rice grain starch synthesis. The *RBE3* gene affected grain amylose content and TGW; furthermore, the *RBE3* gene is negatively correlated with spike size in rice. This transgenic rice is important material for amylose content production in industry.

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