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Research Article

Overexpression of *starch branching enzyme 1* gene improves eating quality in *japonica* rice

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Abstract Eating quality of rice attracts more and more attention from rice-eating consumers in the recent years. Thus, improvement of eating quality of cooked rice has become one of the most important breeding goals in *japonica* rice. Here, the generation of transgenic *japonica* rice with improved eating quality and grain yield are reported. Overexpression of *OsSbe1* gene encoding rice *starch branching enzyme 1* was driven by 35S promoter. Eleven independent homozygous T_3 transgenic lines were characterized and had shown higher palatability (71.2 ~ 72.6) than wild type Gopum (70.4). Moreover, transgenic rice lines showed an increase in 1000-grain weight and number of spikelets per panicle compared with the wild type. The yield of milled rice was 562.8 ~ 596.7 kg/10a in eight T_3 lines, but 542.1 kg/10a in wild type. Gene expression analyses in mRNA transcription and enzyme activity levels suggest that improved eating quality is due to the up-regulation of *OsSbe1* gene.

Keywords Japonica rice, Sbe1, Eating quality, Transgenic, Overexpression

Introduction

Rice is one of the most important crops being the staple food of more than half of the world's population. Each year, large amounts of rice are produced in the world, but most of them are low in nutritional, functional, and eating quality. The eating quality is getting more and more attention from the final rice-eating consumers with the improvement of people's living standards. Because of these demands, the improvement in eating quality of cooked rice has become one of the most important goals in many *japonica* rice breeding programs (Bao et al. 2006; Takeuchi et al. 2007; Lestari et al. 2009; Tian et al. 2009).

The eating quality of rice is mainly influenced by physiochemical properties of starch, which accounts for 76.7 ~ 78.4% of polished rice with 14% moisture content (Huang et al. 1998). Starch is an insoluble glucan composed of two polymers of glucose, amylopectin and amylose, in which amylopectin makes up more than 65% of storage starch in rice endosperm (Nakamura 2002; Zeeman et al. 2010). Amylose is a linear molecule containing α-1, 4-linked glucose units with a small number of branches whereas amylopectin is a large and branched molecule, whose glucosyl residues are linked by α-1, 4-bonds to form chains of between 6 and > 100 glucosyl residues in length. The α-1, 4-linked chains are connected by α-1,6-bonds (branch points). Although its exact molecular architecture is not known, the combination of chain lengths, branching frequency, and branching pattern give rise to a racemose or treelike structure in clusters of chains occurs at regular intervals along the axis of the molecule (Zeeman et al. 2010). Further, amylopectin is required for normal starch granule formation, as evidenced by the fact that varied granule morphologies result from disruptions in amylopectin synthesis. However, amylose content is not a requirement for normal granule formation, as starch granule morphology is similar even with widely varying amylose content (Keeling and Myers 2010). Hence, the composition of amylopectin is the determinant of rice eating quality under certain threshold of protein content and the ratio of amylose to amylopectin (Ong and Blanshard 1995a, 1995b; Juliano 1998; Bao et al. 2006; Lestari et al. 2009).

Most genes and enzymes involved in starch biosynthesis pathway in rice have been described (Nakamura 2002; Tian et al. 2009). Using ADP-glucose as substract, starch is synthesized by soluble starch synthase (SSS), granule-

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bound starch synthase (GBSS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) in rice endosperm. The glucosyl moiety is transferred onto existing glucan chains by starch synthases. *GBSS1* binds tightly to the starch granule and is responsible for amylose synthesis (Denyer et al. 2001). Furthermore, *GBSS1* can elongate amylopectin chains, and form the extra long chains of amylopectin (Denyer et al. 2001; Hanashiro et al. 2008). *SSS1*, *SSS2A* (*SSSII-3*), and *SSS3A* (*SSSIII-2*) preferentially elongate short, medium, and long chains of amylopectin in rice endosperm, respectively (Nakamura et al. 2005; Fujita et al. 2006; Fujita et al. 2007). The branching of amylopectin proceeds concurrently with chain elongation (Nielsen et al. 2002). SBE is the only enzyme which can cut existing α-1,4-glucan chains and transfer the cut segment of six or more glucose units to the C_6 position of a glucosyl residue of another (or the same) glucan chain. Starch branching enzyme has three isoforms in rice. They are *SBE1*, *SBE3* (*QEIIa* or *BEIIb*), and *SBE4* (*QEIIb* or *BEIIa*). Among them, *SBE1* has much higher expression level than *SBE3* and *SBE4* in rice endosperm (Yamanouchi and Nakamura 1992; Tanaka et al. 2004; Dian et al. 2005). When *SBE1* is lacking, amylopectin contains fewer intermediate chains ($16 \leq DP \leq 23$) and long chains ($DP \geq 37$), but more short chains ($DP \leq 12$) (Nakamura 2002; Satoh et al. 2003). Moreover, *SBE1* plays an important role in the synthesis of B_1 chains and B_2-B_3 chains. When *SBE3* is lacking, it leads to a specific decrease in short chains ($DP \leq 13$) with the greatest decrease in chains ($8 \leq DP \leq 11$) (Nishi et al. 2001; Nakamura 2002). Whereas when *SBE4* is lacking, the amylopectin chain profile does not have significant change except that the levels of short chains ($DP \leq 10$) decrease significantly in the amylopectin of the rice leaf sheath where *SBE3* is not expressed (Nakamura 2002). SBE4 activity accounts for about 15~20% of the total SBE activity in rice endosperm, which is similar to the contribution of *SBE3* (Yamanouchi and Nakamura 1992). In parallel, DBE cleaves branch points and is an important determinant of amylopectin structure. It contains isoamylase (ISA) and pullulanase (PUL). *ISA1* and *ISA2* mainly debranch chains with $6 \leq DP \leq 15$, scarcely attack chains with $DP \geq 25$, which mainly express in rice endosperm (Fujita et al. 2003; Wong et al. 2003; Kubo et al. 2005; Utsumiy and Nakamura 2006). Pullulanase debranches short branched chains with $2 \leq DP \leq 4$, partially overlaps with *ISA1* and has much smaller effect than *ISA1*, which mainly expresses in rice endosperm (Ohdan et al. 2005; Fujita et al. 2009). Therefore, the specific pattern of chain-length profiles in amylopectin is mainly controlled by the relative activities of distinct isoforms of SSS, SBE, and DBE in rice endosperm.

The eating quality of rice, a very complex trait mainly evaluated by sensory test or Toyo taste meter (Tanaka et al. 1992; Hwang et al. 2005; Takeuchi et al. 2007; Lestari et al. 2009; Ju et al. 2009: Cho et al. 2010), is controlled by multiple genes. Recent studies identified several QTLs for rice eating quality. Lee (2003) identified four loci for palatability value from Toyo Mito Meter on chromosomes 1, 6, 7, and 12 which affected mostly as minor QTLs by using 179 recombinant inbred lines (RILs) derived from a *japonica* rice cross between Ilpumbyeo and YR15965-Acp33. However, the amylose and protein content are not related with QTLs for palatability value on chromosome 6 (*Waxy* locus). Takeuchi et al. (2007) revealed one locus for palatability from sensory test on chromosome 6 by using 182 backcross inbred lines (BILs) derived from a cross of Koshihikari/2*Kasalath (*indica*), and five loci for palatability from sensory test on chromosomes 1, 3, 6, 10 by using 39 CSSLs (Ebitani et al. 2005). Wada et al. (2008) identified eight loci for palatability value from sensory test on chromosomes 1, 2, 6, 7, 8, and 12 by using 92 recombinant inbred lines (RILs) from *japonica* rice cross between Moritawase and Koshihikari.

Relationships between gene structure and enzyme activity related to starch biosynthesis and rice eating quality properties have been evaluated to accelerate high eating quality rice breeding. Tian et al. (2009) reported that *Waxy* and *ALK* (*SSS2A*) are central in determining rice eating and cooking quality by affecting amylose content, gel consistency, and gelatinization temperature whereas both *ISA* and *SBE3* affect gel consistency and gelatinization temperature. Further studies showed that several minor genes are specific for each property: *SSS3A*, *AGPlar* (ADP-glucose pyrophosphorylase large subunit gene), *PUL* (Pullulanase gene), and *SSS1* for amylose content, *AGPiso* (ADP-glucose pyrophosphorylase large subunit isoform gene) for gel consistency, and *SSS4B* for gelatinization temperature. The correlations among amylose content, gel consistency, and gelatinization temperature were caused by the joint action of these associated genes. Sun et al. (2011) found that SNPs and InDels from *SBE1*, *SBE3*, *SSS1*, and *SSS2A* could differentiate high or intermediate palatability *japonica* rice varieties from low palatability *japonica* rice varieties. Zhao et al. (2005) revealed that SSS activity at the early grain filling stage was negatively correlated with gel consistency and alkali digestion value, and positively correlated with amylose content. Activities of SSS at middle and late grain filling stages and SBE at the late grain filling stage were positively correlated with gel consistency and alkali digestion value,

and negatively correlated with amylose content. Shen et al. (2006) reported that the activities of AGPase and SBE at 12, 24, and 30 days after heading were correlated with palatability, and the activities of AGPase, SSS, and SBE were correlated with RVA pasting properties. Sun et al. (2011) revealed that activity of SBE at 25 days after flowering was positively correlated with palatability, whereas activity of GBSS1 at 10 and 15 days after flowering was negatively correlated with palatability. To date, a number of studies have suggested that overexpressions of *SBE1* (Chen 2003; Kim et al. 2005; Wu et al. 2006), *SBE3* (Chen 2003), and *SSS2A* (US 20090106863A1), and inhibition expression of *GBSS1* (Terada et al. 2000) may improve eating quality in rice to some extent. However, despite a number of such efforts to develop high eating quality rice plants, very few have shown an improvement in overall eating quality except reduced amylose content and changed in the degree of polymerization of amylopectin chains.

In this study, the overexpression of *OsSbe1* gene driven by the strong 35S promoter in *japonica* rice is reported. The transgenic rice lines showed higher eating quality score compared with the wild type under normal field condition. In addition, it exhibited an increase in spikelets per panicle and 1000-grain weight, resulting in an increase grain yield. Moreover, the improved eating quality is due to the up-regulation of *OsSbe1* gene as evidenced by the increase in the mRNA transcription and enzyme activity.

Materials and Methods

Construction of plant expression vector

The pCAMBIA1300 vector (Cambia) which contains the hygromycin phosphotransferase gene (*HPT*) under the control of 35S promoter was used to enable hygromycin-based plant selection. The open reading frame of *OsSbe1* gene (GenBank Accession No. AF136268) was amplified from Nipponbare (*Oryza sativa* L. ssp. *japonica*) cDNA with primer pair SBE1-Xba1-Fw/SBE1-Xba1-Rv (Table 1), and inserted into the multiple cloning sites *Xba*I of the modified pCAMBIA1300 vector carrying the 35S promoter and the nopaline synthase (NOS) terminator. The final expression vector is named as pCSBE1, and it is shown in Figure 1. The pCSBE1 expression vector was introduced into *Agro-bacterium tumefaciens* strain LBA4404 by electroporation using a MicroPulser electroporation system (Bio-Rad Laboratories, Hercules, CA), and cultured on AB medium containing 50 mg/L kanamycin sulfate for 3 days at 30℃ in the dark.

Table 1 Sequences and amplicon length of primer pairs used in the experiment

Primer name	Sequence (5'-3')	Amplicon length (bp)
SBE1-Xba1-Fw*	TCTAGAATGCTGTGTCTCACCTCC	2,475
SBE1-Xba1-Rv	TCTAGATCATTTGCAGTCTTCGTCAG	
35S-Fw	CGCACAATCCCACTATCCTT	373
SBE1-Rv	GAAATTGGTCTTGACCTTTCCA	
HPT-Fw	ATTTGTGTACGCCCGACAGT	259
HPT-Rv	GGGATTCCCAATACGAGGTC	
SPS-Fw	TTGCGCCCTGACGGATAT	81
SPS-Rv	CGGTTGATCTTTTCGGGATG	
SBE1-F1	CTACCATCAACCGTGGCATT	182
SBE1-R1	GTCGACAAGGCTCCACTGAC	
ACT-F1	ATCCTTGTATGCTAGCGGTCGA	118
ACT-R1	ATCCAACCGGAGGATAGCATG	

*SBE1, starch branching enzyme 1; 35S, cauliflower mosaic virus 35S RNA gene promoter; HPT, hygromycin phosphotransferase; SPS, sucrose phosphate synthase; ACT, actin.

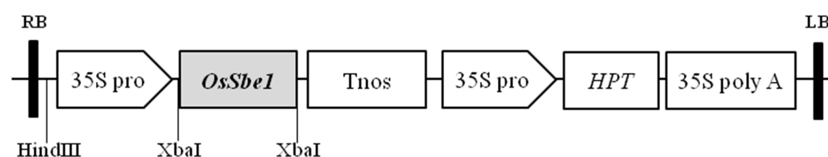

Fig. 1 The pCSBE1 expression vector used in *japonica* rice transformation

Production of transgenic rice plants

The transformation was carried out using the method of Kim et al. (2007) with some modifications. Mature seeds of the *japonica* rice cultivar Gopum were dehulled and sterilized with 70% ethanol for 5 min prior to washing three times in distilled water (10 ml seeds per 50 ml tube). These seeds were further sterilized with 30 ~ 35 ml of 2 ~ 3% sodium hypochlorite containing one drop of Tween 20 per 50 ml for 15 min by strong shaking then washed five times in distilled water. This step was repeated once without Tween 20. The sterilized seeds were washed 20 times in distilled water, dried in the cleanbench for 30 min, and then inoculated on embryogenic callus induction (2N6) medium (Chu et al. 1975) in the dark at 28℃ for four weeks. Bright yellow callus with 1 ~ 2 mm size were selected and transferred onto 2N6-AS medium and precultured for three days. The LBA4404/pCSBE1 culture was suspended in AAM-AS medium to yield an optical density at 650 nm wavelength (OD_{650}) of approximately 1.5. Precultured seeds were immersed in the *Agrobacterium* suspension by gently inverting the tube for 30 min, and then blotted dry with sterilized filter papers to remove excess bacterium. These seeds were transferred onto a sterilized filter paper moistened with 0.5 ml of AAM-AS and covered on co-culture (2N6-AS) medium. After co-cultivation at 26.5℃ for 1 day, and at 23.5℃ for 3 days in the dark, the seeds were washed 12 times in distilled water containing 500 mg/L cefotaxime to remove *Agrobacterium.* The seeds were rapidly blotted dry on a sterilized filter paper and cultured on transgenic callus selection (2N6-CH) medium for two weeks under continuous light at 27℃. Proliferating calli arising from the scutellum were transferred onto shoot regeneration (MSR-CH) medium under continuous light at 27℃ for 3 ~ 4 weeks. Plantets arising from the calli were transferred onto root formation (MSO-CH) medium to induce roots.

Cultivation and investigation of transgenic rice

Regenrated plants in MSO-CH medium were transplanted in greenhouse, and T_1 seeds were harvested from October 2007 to March 2008. T_1 and wild type rice seeds were sterilized and sown in ½ Murashige and Skoog (MS) solid medium (2.165 g/L MS medium) (Murashige and Skoog 1962) including vitamins (Cat. No. M0222, Duchefa Biochemie B.V.), 10 g/L sucrose, 8 g/L Bacto agar, pH 5.8) containing 50 mg/L hygromycin at 28℃ for 7 days. The germinated T_1 seeds were transplanted to seedling-growing tray, and then grown in greenhouse for 3 ~ 4 weeks. Subsequently, seedlings were transplanted in the experimental farm of Chungbuk National University on June 1, 2008. At tillering stage, young leaves of transgenic lines and wild type were sampled for genomic DNA PCR analysis using primer pairs of *OsSbe1* and *HPT* gene (GenBank Accession No. AF234296).

Good phenotypes with low/middle amylose content T_2 seeds (513 lines) were planted in the experimental farm on June 1, 2009. The fertilizers of N-P_2O_5-K_2O were applied at the level of 90-45-47 kg/ha. Other management practices were followed. Data on agronomic traits including days to flowering, plant height, culm length, panicle length, and panicle number per plant from 193 T_2 lines with good phenotype and wild type were investigated. Spikelets per panicle and 1000-grain weight (unpolished rice) were also investigated. The eating quality parameters of selected 52 T_3 seeds with good phenotype and wild type seeds were measured. Moreover, selected T_3 seeds were planted in the experimental farm of Chungbuk National University in 2010 and sampled for transgene copy number, mRNA expression, and SBE enzyme activity analyses.

Apparent amylose content determination

The apparent amylose content in T_2 unpolished rice was determined as described by Mei et al. (2007) and Stawski (2008) with some modifications. Ten mg of fine ground unpolished rice powder was weighed and put into a 20 ml test tube. The rice powder was dispersed in 100 µl of 100% ethanol, mixed well, and then 900 µl of 1 mol/L NaOH was added with swirling to improve dispersion. The contents were heated to boiling in 100℃ waterbath for 30 min until the starch completely gelatinized. The test tube was cooled, mixed on a vortex mixer, and filled to the 10 ml mark with distilled water. Duplicate 500 µl of sample was taken into a new 20 ml test tube containing 5 ml of distilled water. The sample was neutralized with 100 µl of 1 mol/L acetic acid, then 200 µl of fresh iodine-potassium iodide solution (2 g KI + 100 ml distilled water + 0.2 g I_2) and 4.2 ml of distilled water were added. The tubes were mixed on a vortex mixer, and then incubated at 30℃ for 30 min in the dark, and the absorbance of duplicate subsamples was measured at 620 nm using a spectrophotometer.

The standard curve was set up using 1 mg/ml standard amylose (Cat. No: A0512-5G, Sigma-Aldrich) and 1 mg/ml standard amylopectin (Cat. No: 10118, Sigma-Aldrich) solution from potato. A series of amylose content solutions were prepared: 18 ml of standard amylopectin solution plus 2 ml of 0.09 mol/L NaOH (0%), 1 ml of standard

amylose solution plus 17 ml of standard amylopectin solution plus 2 ml of 0.09 mol/L NaOH (5%), 2 ml of standard amylose solution plus 16 ml of standard amylopectin solution plus 2 ml of 0.09 mol/L NaOH (10%), 3 ml of standard amylose solution plus 15 ml of standard amylopectin solution plus 2 ml of 0.09 mol/L NaOH (15%), 4 ml of standard amylose solution plus 14 ml of standard amylopectin solution plus 2 ml of 0.09 mol/L NaOH (20%), 5 ml of standard amylose solution plus 13 ml of standard amylopectin solution plus 2 ml of 0.09 mol/L NaOH (25%), etc. The blank solution was made using 500 μl of 0.09 mol/L NaOH to replace the 500 μl of gelatinized rice sample, and the other procedures were same as sample and standard amylose content samples. The reference used is Gopum whose apparent amylose content is 18%. Three replications for each sample were used.

RNA extraction and first-strand synthesis of cDNA

Total RNAs were isolated from seed endosperms using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instruction with some modifications. About 100 mg of frozen ground seeds powder was placed into cold 1.5 ml tube, and then 600 μl of RLC buffer was added. The mixture was immediately mixed by vortexing, and centrifuged at 13,000 rpm for 2 min. The supernatant was rapidly transferred into lilac spin column, then centrifuged at 12,000 rpm for 2 min. 280 μl of 100% ethanol was added into the flowthrough, mixed 4 ~ 5 times by pipetting and immediately transferred into pink RNeasy spin column. After centrifuging at 10,000 rpm for 1 min, 700 μl of RW1 buffer was added into the pink spin column. Centrifugation was carried out at 10,000 rpm for 1 min, and then 500 μl of RPE buffer was used to wash RNA twice at 10,000 rpm for 1 min. After 2 min of centrifugation at 10,000 rpm, 50 μl of RNase-free water was added into the pink spin column in 1.5 ml tube, and centrifuged at 10,000 rpm for 1 min. The RNA concentration was detected with Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. USA), and stored at -80℃. Total RNAs were cleaned using DNase 1 kit (Cat. No: 18068-015, invitrogen), and the first-strand cDNA synthesis was achieved by reverse transcription of mRNA using Oligo(dT)$_{20}$ primer and SuperScript^{TM} III Reverse Transcriptase (Cat. No: 18080-051, Invitrogen).

Transgene copy number

Genomic DNA was isolated from young leaf tissue of wild type and 16 T_3 lines. Quantitative genomic DNA PCR was carried out using SPS-Fw/SPS-Rv and HPT-F1/HPT-R1 (Table 1). The sucrose phosphate synthase gene (*SPS*) was used as endogenous reference gene for rice (Ding et al. 2004; Yang et al. 2005; Jiang et al. 2009). The amplication conditions were as follows: 8 min at 95℃ followed by 39 cycles of 33 sec at 94℃, 32 sec at 55℃, 22 sec at 72℃, and final extension of 5 min at 72℃. To generate standard curve of *HPT*, pCAMBIA 1300 plasmid DNA solutions were serially diluted to final concentrations of 10^9, 10^8, 10^7, 10^6, 10^5, and 10^3 copies/5 μl (5 μl per reaction). As for standard curve of *SPS*, wild type rice genomic DNA solutions were serially diluted to final concentration of 115500, 46200, 11550, 4620, and 1155 copies/5 μl (5 μl per reaction).

To estimate the transgene copy number, a relative quantitative method that combined two absolute quantification reactions was used. One is for the target specific gene, and the other is for the endogenous reference gene (Ahmed 2002; Laura et al. 2002). The quantified result of *HPT* transgene and *SPS* reference gene for each line was calculated and compared as described by Opticon 2 system operations manual and Yang et al. (2005).

Semi-quantitative RT-PCR and quantitative real-time RT-PCR analyses

The protein sequence of starch branching enzyme 1 was aligned using the NCBI/BLAST website, and the clustalw (DDBJ/CLUSTALW site) of protein sequences from 15 species was carried out. Using DNA sequences of conserved domains, primers were designed with Primer3 online software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and primer analysis was carried out using Oligo 6 software. The primer pair was SBE1-Fw and SBE1-Rv. The *Actin-1* gene (GenBank Accession No. AK-071586) was employed as internal reference. The primer pair was ACT1-Fw and ACT1-Rv (Table 1).

Semi-quantitative RT-PCR was carried out in 20 μl of reaction mixture containing 50 ng of first-strand cDNA, and 5 pmol of SBE1-Fw/SBE1-Rv primer, and 0.5 unit of Takara Ex-Taq (Takara Bio Inc.). PCR was performed at 94℃ for 4 min, followed by 28 cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 30 sec. Amplified products were resolved on 1.5% agarose gel. The expression of *Actin-1* gene served as an internal reference.

Quantitative real-time PCR was performed on the DNA Engine Opticon 2 (MJ Research, Waltham, MA) instrument in a final reaction volume of 20 μl with SYBR Green, a

fluorophore which binds to all double-strand DNA (F-410L, qPCR kit from FINNZYMES, Finland). The SBE1-Fw/ SBE1-Rv primer pair was used in this experiment. PCR conditions were as follows: 8 min at 95℃ followed by 39 cycles of 33 sec at 94℃, 32 sec at 55℃, 22 sec at 72℃, and final extension of 5 min at 72℃. Finally, the relative quantification of gene expression was analyzed using the 2-ddCt method (Livak and Schmittgen 2001) by normalization to internal *Actin-1* mRNA levels.

Starch branching enzyme activity assay

Enzyme activity of starch branching enzyme including *SBE1*, *SBE3*, and *SBE4* isoforms in rice grains was assayed as described by Yamanouchi and Nakamura (1992) with some modifications. Fifteen frozen rice endosperms were weighed and homogenized in 4 ml of cold extraction buffer (50 mM HEPES-NaOH pH 7.4, 4 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 12.5% (v/v) glycerol) contained in a precooled mortar with a pestle on ice. The homogenate was transferred into a precooled 40 ml tube, and 6 ml of extraction buffer was added for two times to clean the mortar. 40 ml tubes were centrifuged at 15,000 rpm at 2℃ for 20 min, and supernatants were filtered through a filter paper (0.45 μm, Whatman, Whatman International LTD). The filtrates were used for enzyme preparations.

Starch branching enzyme was assayed by monitoring stimulation of α-glucan synthesis from glucose-1-phosphate by rabbit muscle phosphorylase A (Hawker et al. 1974; Nakamura et al. 1989). 100 μl of enzyme preparation was added into 100 μl of cold reaction buffer (50 mM HEPES-NaOH pH 7.0, 50 mM glucose-1-phosphate, 2.5 mM AMP, 1.2 units of phosphorylase a from rabbit muscle), and was incubated at 30℃ for 30 min. The reaction was terminated by adding 50 μl 1M HCl, and mixed with 500 μl of dimethylsulfoxide. 700 μl of fresh iodine-potassium iodide solution (2 g KI plus 200 ml distilled water plus 0.2 g I_2) was added into the solution, and then incubated at 30℃ for 30 min in the dark. The SBE activity was determined by measuring the absorbance at 540 nm in a UV spectrophotometer (Shimadzu). The controls were prepared as follows: 100 μl of enzyme preparation was added into 50 μl 1M HCl, mixed well, and 100 μl of cold reaction buffer was added into the mixture. The other steps were same as reaction samples. The blank solution was made using 100 μl of cold extraction buffer to replace 100 μl enzyme preparation and the other steps were same as reaction samples. Three replications were carried out in this experiment.

The starch branching enzyme activity was calculated as follows: SBE activity=$\triangle A \times 100 \times V_R \times V_T/(T \times N \times d \times V_S \times V_S)$; $\triangle A$, absorbance of samples at 30 min minus absorbance of samples at 0 min; T, time (minute); N, grain number; d, light path in cm; 100, 0.01 optical density as 1 unit which has similar function as ε or %E; V_R, reaction volume (enzyme sample volume + working reagent volume + stop solution volume) (ml); V_S, enzyme sample volume (ml); V_T, total enzyme sample volume (ml). So SBE activity=$\triangle A \times 100 \times 1.45 \times 10/(30 \times 15 \times 1 \times 0.1 \times 0.1)=322.22 \times \triangle A$(Units $grain^{-1}$ min^{-1}).

Eating quality traits determination

The rice grains were dehulled and milled to 91% yield, and eating quality was evaluated as described by Peng et al. (2006) and Lestari et al. (2009). Palatability (overall eating quality) was detected by a rice taste measuring system (Toyo taste meter, model MA-90) according to the manufacture's instruction (TRCM Co.) (Toyo Rice Polishing Machine Factory, Japan). Protein content was determined by the micro-kjeldahl method (Cunniff 1995), and calculated using total nitrogen multiplied by the conversion coefficient 5.95 after the determined value. The amylose content of milled rice in T_3 seeds was measured using the relative absorbance of starch-iodine color in a digested solution of 100-mesh rice flour as described by Perez and Juliano (1978). The alkali digestion value (ADV) was estimated using the alkali digestion test and digestion scores (Little et al. 1958).

RVA pasting properties were detected with a Rapid Visco Analyzer (RVA) according to the manufacture's instruction (NewPort Sci. Co., Australia). Rice starch paste profile was described by seven parameters. They were as follows: peak viscosity (PV), through viscosity or hot paste viscosity (HPV), final viscosity or cool paste viscosity (CPV), breakdown viscosity (BDV=PV-HPV), setback viscosity (SBV=CPV-PV), consistency viscosity (CTV=CPV-HPV), and pasting temperature as described by Bao and Xia (1999) and Shen et al. (2006). All the viscosity parameters were expressed in rapid visco units (RVU).

Statistical analysis

The frequency distribution of various traits including agronomy and eating quality was carried out using Microsoft Excel 2007. The variance for various agronomic traits and eating quality parameters was conducted using Statistix software version 8.0 (http://www.statistix.com/). T-tests for

agronomic traits, grain yield components, and eating quality between wild type and transgenic line groups were carried out using Microsoft Excel 2007.

Results

Generation of transgenic rice

Transformation of embryogenic calli of the *japonica* rice cultivar Gopum with the expression vector pCSBE1 resulted in the production of 165 independent transgenic plants, in which 134 T_0 plants produced seeds. Of the 134 transgenic plants, 110 T_1 seeds showed resistance to hygromycin. These lines were planted in the field, and genomic DNA was isolated from young leaf tissues. To further verify the gene integration, PCR analysis was done using HPT-Fw/HPT-Rv and 35S-Fw/SBE1-Rv primer pairs (Table 1, Fig. 2). The results showed that *HPT* and *OsSbe1* were integrated into rice genome in 1,005 out of 1,065 T_1 plants. Most of the T_1 plants displayed the same or similar phenotype as wild type, but some lines produced mutations such as purple stem, long awn spikelet, erect panicle, dwarf and few tillers (data not shown). The confirmed and good phenotype lines were advanced to several generations to ensure that transgene expression was heritable and stable. After four generations of self-pollination, there were no obvious changes in grain color and seed shape.

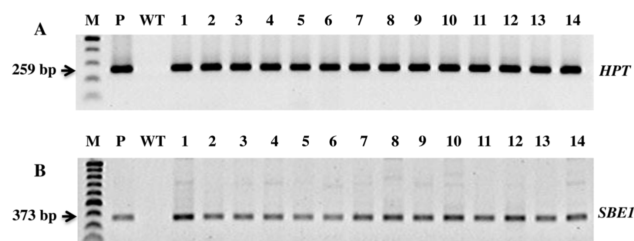

Fig. 2 PCR detection of *HPT* (A) and *OsSbe1* (B) in T_1 plants transformed pCSBE1 expression vector by *Agrobacterium*-mediated method. M, molecular weight size marker; P, pCSBE1 plant expression vector; WT, wild type; 1 ~ 14, T_1 plants

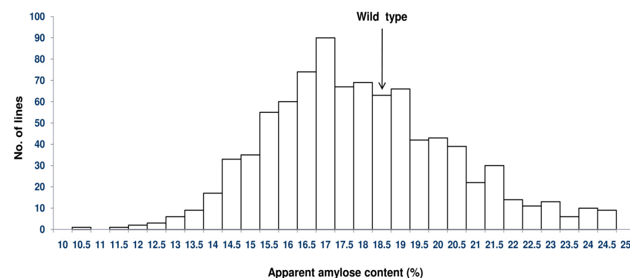

Fig. 3 Distribution of apparent amylose content on unpolished rice of 890 T_2 lines

Analysis of T_2 and T_3 seeds

The hypothesis was that successful transformation of starch branching enzyme 1 gene (*OsSbe1*) would give rise to lower or similar apparent amylose contents and better physical composition of amylopectin in transgenic plants compared with the wild type. Therefore, as a first approach to assess the effect of transgene expression on rice seeds, the apparent amylose contents of 890 T_2 unpolished rice were detected (Fig. 3). It varied from 10.6 ~ 25.0% in 890 T_2 seeds, and 18% in the wild type. Of the 890 T_2 seeds, 453 seeds showed lower apparent amylose content compared to the wild type in which 72 T_2 seeds were lower than 15%. Whereas, the apparent amylose contents in 197 T_2 seeds were higher than 20%, which could be caused by cosuppression of endogenous and exogenous genes, gene silencing, insertion site or positional effect.

Moreover, T_2 lines below 18% (453) and around 18% (46) apparent amylose content in unpolished rice were grown in the field. T_3 lines and wild type panicles at 15 days after flowering were randomly sampled, and semi-quantitative RT-PCR was conducted (Fig. 4). The *OsSbe1* gene in the transgenic lines showed higher expression level of mRNA than that in wild type indicating the successful overexpression of the gene.

Variation in eating quality parameters and grain yield components of transgenic rice lines

To determine the changes in the eating quality of transgenic rice, T_2 plants with good phenotype were selected, and the palatability of 52 T_3 seeds was detected by a rice taste measuring system (Table 2, Fig. 5). The palatability was 61.1 ~ 72.6, with the coefficient of variation of 4.3%, whereas 70.4 in the wild type. This indicates that a wide variation exists in the transgenic lines, and can be used to

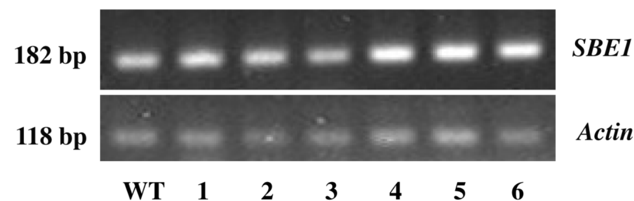

Fig. 4 Semi-quantitative RT-PCR of *OsSbe1* gene in wild type and T_3 seeds sampled at 15 days after flowering. WT, wild type; 1 ~ 6, T_3 panicles of CBRT0701-4-12-2, CBRT0701-71-8-10, CBRT0701-58-9-3, CBRT0701-39-12-4, CBRT0701-130-11-5, and CBRT0701-163-8-6

Table 2 Eating quality parameters in wild type and 52 T_3 lines under normal field condition

Parameters	WT	T_3 lines				
	Mean ± SD	Mean ± SD	Range	CV (%)	Skewness	Kurtosis
P[a]	70.4 ± 0.57	67.6 ± 2.93	61.1-72.6	4.33	-0.17	-0.58
AAC (%)	18.0 ± 0.01	18.2 ± 0.69	16.9-19.8	3.77	0.09	-0.74
PC (%)	6.13 ± 0.07	6.47 ± 0.33	5.91-7.80	5.12	1.17	3.43
ADV[b]	6.3 ± 0.06	6.45 ± 0.09	6.3-6.6	1.42	0.17	-0.81
PV[b]	276.1 ± 7.21	251.7 ± 42.9	142.0-325.4	17.1	-0.88	-0.17
HPV[b]	63.5 ± 9.20	125.8 ± 36.9	29.9-163.7	29.3	-1.26	-0.02
CPV[b]	269.9 ± 9.88	234.9 ± 40.3	124.4-281.8	17.2	-1.23	0.11
BDV[b]	112.6 ± 1.96	125.8 ± 15.6	94.2-170.4	12.4	0.34	0.02
SBV[b]	-6.17 ± 2.64	-16.8 ± 15.1	-60.0 to 22.5	90.2	-0.30	0.44
CTV[b]	106.4 ± 0.68	109.1 ± 8.4	94.6-127.1	7.67	0.53	-0.65
PT (℃)	68.1 ± 0.04	68.1 ± 0.04	68.1-68.2	0.05	0.36	-0.57

[a]P, palatability value; AAC, apparent amylose content; PC, protein content; ADV, alkali digestion value; PV, peak viscosity; HPV, hot pate viscosity; CPV, cool paste viscosity; BDV, breakdown viscosity; SBV, setback viscosity; CTV, consistency viscosity; PT, pasting temperature (℃); CV, coefficient of variation; WT, wild type; [b]RVU, rapid visco unit.

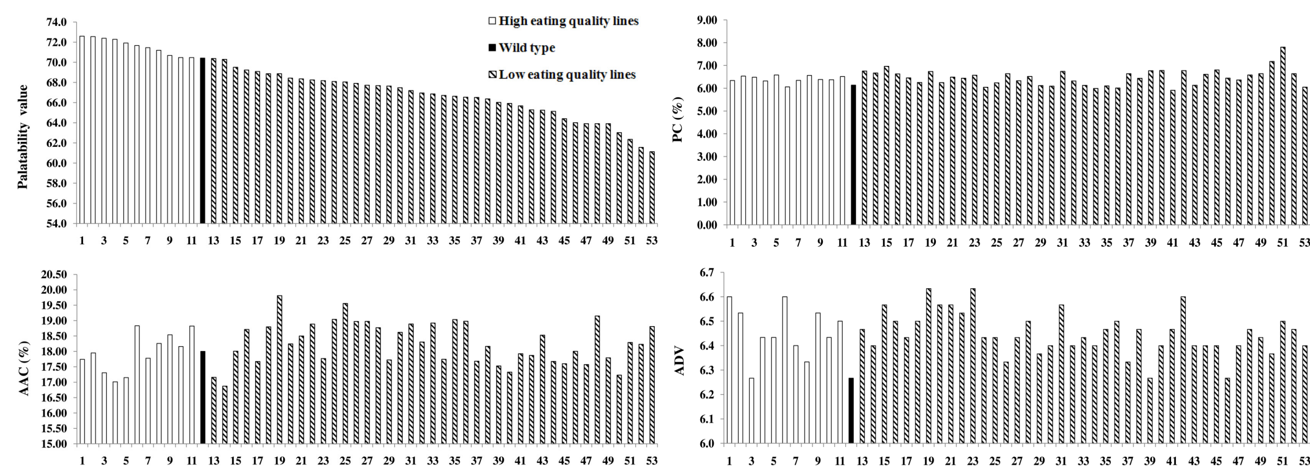

Fig. 5 Distribution of palatability value and apparent amylose content (AAC) in wild type and 52 T_3 lines

develop new rice varieties with high eating quality using transgenic lines as materials. Moreover, the apparent amylose content ranged from 16.9 to 19.8% with an average of 18.2%, which was similar to the wild type. Further, the protein content was 5.9～7.8% with an average of 6.5% whereas the alkali digestion value was 6.3～6.6 indicating that the gelatinization temperature was low. Compared with the wild type, the pasting properties were variable in T_3 lines. The breakdown viscosity was 94.2～170.4 RVU (rapid visco unit) with an average of 125.8 RVU, whereas the wild type was 112.6 RVU. The setback viscosity varied from -60.0 to 22.5 RVU with an average of -16.8 RVU, while -6.17 RVU in the wild type (Table 2).

The agronomic traits in $T_{2:3}$ lines were also measured (Table 3). The days from seeding to flowering in T_2 lines were 107～115 days with an average of 112 days, while 113 days in the wild type. Plant height was 83.7～113.5 cm, with a coefficient of variation of 4.14% and a mean of 100.1 cm in the transgenic, whereas 102 cm in the wild type. The panicle length was 18.6～26.6 cm with a coefficient of variation of 5.73%. Average panicle length in T_2 lines was 22.0 cm, while 22.6 cm in the wild type. The number of panicles per plant were 8～16 with with an average of 11 compared to the wild type of 10. Further investigation of the 1000-grain weight showed 20.3～25.1 g, with an average of 22.7 g, while 21.0 g in the wild type. Collectively, most of the agronomic traits were relatively higher in the transgenic plants compared to the wild type Gopum. Overall, eight lines with high palatability (71.2～72.6) were selected to use as breeding lines. They were

Table 3 Agronomic traits in wild type and 52 $T_{2:3}$ lines under field condition

Traits	WT	$T_{2:3}$ lines				
	Mean ± SD	Mean ± SD	Range	CV (%)	Skewness	Kurtosis
Days from seeding to flowering	113 ± 0.52	112 ± 1.16	107-115	1.04	-0.4	1.01
Plant height (cm)	102.0 ± 1.96	100.1 ± 4.15	83.7-113.5	4.14	-0.5	2.52
Culm length (cm)	71.2 ± 2.25	71.0 ± 4.70	56.5-81.3	6.62	-0.22	-0.13
Panicle length (cm)	22.6 ± 1.41	22.0 ± 1.26	18.6-26.6	5.73	-0.17	0.61
Panicles per plant	10 ± 1.47	11 ± 1.46	8-16	13.25	0.28	0.44
1000-grain weight (g)[a]	21.0 ± 0.14	22.7 ± 1.08	20.3-25.1	4.77	-0.28	-0.13

[a]1000 unpolished rice grain weight.

Table 4 Agronomic traits, grain yield components, and eating quality in wild type and 11 $T_{2:3}$ lines under field condition

T3 lines	Flowering date	Plant Height (cm)	Panicle length (cm)	Panicle fertility (%)	1000-grain weight (g)[a]	Spikelets /panicle	Panicles /plant	Milled rice yield (kg/10a)	Palatability
WT	Aug. 16	102.5	23.2	91.8	21.0	165	10	542.1	70.4
CBRT0701-57-18-3	Aug. 16	104.7	22.7	83.8	23.0	180	10	591.2	72.6
CBRT0701-39-9-2	Aug.16	100.9	20.6	84.5	23.0	194	9	578.3	72.5
CBRT0701-44-18-5	Aug.15	104.4	22.9	83.0	21.8	190	10	585.9	72.4
CBRT0701-67-5-9	Aug.17	101.1	23.0	88.2	20.8	200	9	562.8	72.3
CBRT0701-50-2-6	Aug.15	106.1	23.2	87.8	23.1	185	9	575.5	71.9
CBRT0701-100-4-5	Aug.15	110.7	22.0	80.2	23.6	185	10	596.7	71.7
CBRT0701-67-12-2	Aug.17	103.6	23.5	89.1	22.5	192	9	590.4	71.5
CBRT0701-71-8-10	Aug.14	105.9	23.6	89.5	21.3	179	10	581.5	71.2
CBRT0701-61-1-2	Aug.16	105.2	24.5	86.1	22.0	201	9	584.0	70.7
CBRT0701-114-8-3	Aug.14	103.5	23.6	80.4	20.7	194	10	550.2	70.5
CBRT0701-67-4-6	Aug.16	102.0	22.8	84.0	23.8	183	9	561.1	70.5
P value[b]	0.228	0.076	0.214	2.5×10-9	0.004	0.022	0.133	0.029	0.048

[a]1000 unpolished rice grain weight; [b]P value, T-test between wild type and transgenic line groups.

CBRT0701-57-18-3, CBRT0701-39-9-2, CBRT0701-44-18-5, CBRT0701-67-5-9, CBRT0701-50-2-6, CBRT0701-100-4-5, and CBRT0701-67-12-2 lines (Table 4). The protein content of these lines was similar to the wild type. However, the alkali digestion value was higher (Fig. 5). More, the estimated grain yield (milled) was 550.2 ~ 596.7 kg/10a, while 542.1 kg/10a in the wild type. Further analysis showed that there was no significant differences for flowering date and panicle length between wild type and transgenic lines whereas significant differences were found in the eating quality, milled rice yield, panicle fertility, 1000-grain weight, spikelets per panicle, panicles per plant, and panicle length (Table 4).

Increase in *OsSbe1* gene expression associated with eating quality improvement of transgenic rice lines

To test whether the variability in overall eating quality was due to differences in transgene copy number, quantitative PCR analysis of genomic DNA was performed on high and low palatability T_3 lines (Fig. 6). Results showed that transgenic lines had a variable transgene copy number, ranging from 1 to 11 copies. High eating quality lines was 1 ~ 4 copies whereas, the low eating quality lines was 1 ~ 11 copies. Thus, the copy numbers were not significantly correlated with rice eating quality (Table 5) indicating that it may not affect the overall eating quality.

To further understand the variability in the transgenic lines and to determine the basis for the increased palatability, the RNA expressions of *OsSbe1* in the wild type and selected high eating quality T_4 seeds at 10 and 15 days after flowering were analyzed by quantitative real-time PCR (Fig. 7). Results showed that the mRNA expressions of *OsSbe1* at 10 and 15 days after flowering were increased in the high eating quality lines compared with wild type. Further analysis on the activities of starch branc-

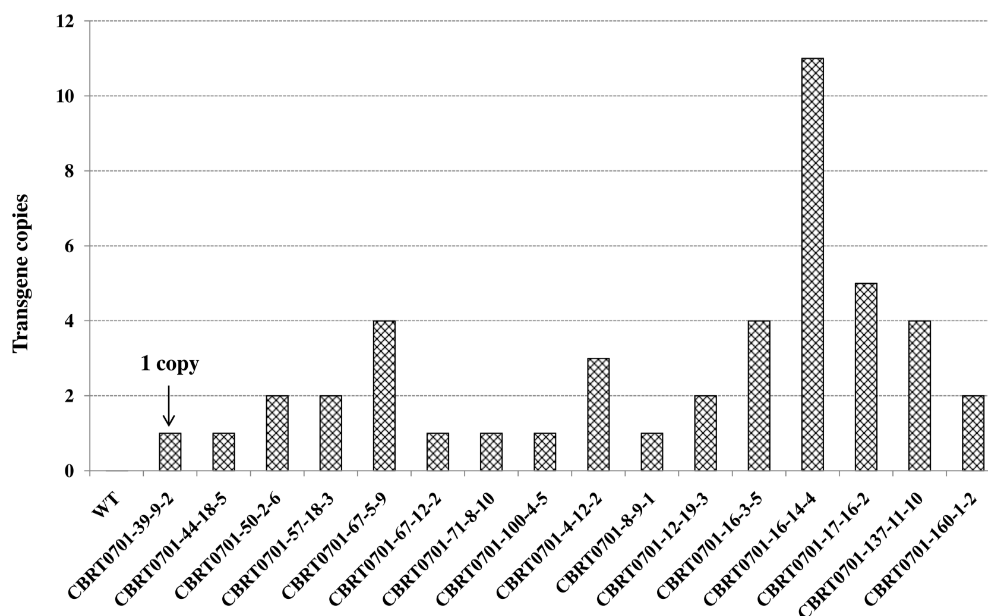

Fig. 6 Transgene copies in wild type and T_3 lines detected by quantitative real-time genomic DNA PCR. CBRT0701-39-9-2-CBRT0701-100-4-5 lines had high eating quality (see Table 4), while CBRT0701-4-12-2-CBRT0701-160-1-2 lines had low eating quality whose palatability score was 63.9, 63.9, 65.7, 61.6, 64.4, 65.1, 63.0, and 61.1, respectively

Table 5. Correlation between palatability score and transgene copies, and starch branching enzyme activity, respectively

Parameter	Palatability score	Probability
Transgene copies	-0.42	0.1087
SBE activity	0.73	0.0263

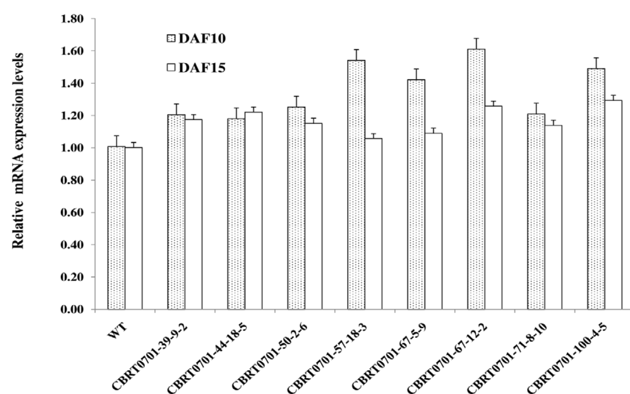

Fig. 7 The transcriptional levels of *OsSbe1* gene in wild type and T_4 seeds detected by quantitative RT-PCR. The panicles derived from above T_3 lines and wild type were collected at 10 and 15 days after flowering, respectively. Data were normalized using the rice *Actin* gene. Values are means (± SD) of three replications

hing enzyme in the wild type and transgenic line seeds at 25 days after flowering, showed that high eating quality lines had a higher activity than wild type which was positively and significantly correlated with rice eating quality (Table 5, Fig. 8).

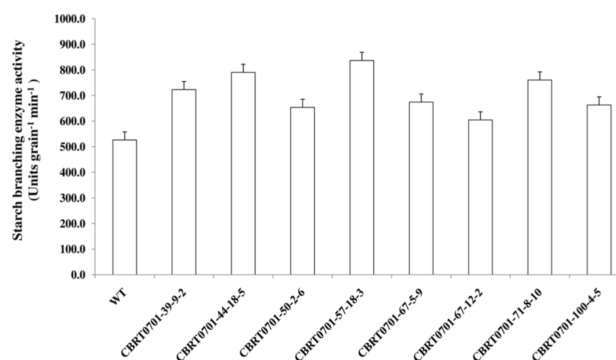

Fig. 8 Activities of starch branching enzyme in wild type and T_4 seeds sampled at 25 days after flowering. The T_4 panicles were derived from above T_3 lines, respectively. Values are means (± SD) of three replications

Discussion

Rice eating quality is becoming more and more popular for rice-eating consumers and researchers. It is a very complex trait, which is mainly determined by the composition or fine structure of amylopectin under certain threshold of amylose content and protein content (Reddy and Ali 1993; Ong and Blanshard 1995a, 1995b; Juliano 1998; Baxter et al. 2004; Sun et al. 2011). Among enzymes related to starch biosynthesis, soluble starch synthase, granule bound starch synthase, starch branching enzyme, and starch de-branching enzyme play important roles in amylopectin biosynthesis (Nakamura 2002). Furthermore, starch bran-

ching enzyme is the only enzyme that can introduce α-1, 6-glucosidic linkage into α-polyglucans in plants (Nakamura 2002), which significantly contributes to the fine structure of amylopectin and eating quality of *japonica* rice (Satoh et al. 2003; Kim et al. 2005; Hong 2006; Sun et al. 2011).

The starch branching enzyme 1-deficient rice mutant endosperm exhibited the normal phenotype, but the mutation apparently altered the fine structure of amylopectin (Satoh et al. 2003). Using 217 doubled haploid lines derived from a *japonica* cross between Ilpum and Palgong, the relationships between amylopectin polymerization and eating quality was positively and significantly correlated with chains of DP 7, 8, 9, 32, and 33, but negatively and significantly correlated with chains of DP 15 ~ 19 (Hong 2006). To test whether improved amylopectin polymerization can serve as the source of enhanced levels of palatability in *japonica* rice, the *rice starch branching enzyme 1* gene (*OsSbe1*) was introduced into *japonica* rice cultivar Gopum driven by 35S promoter. The overexpression of *OsSbe1* gene can improve the eating quality and grain yield in *japonica* rice.

The apparent amylose contents of unpolished rice were 10.6 ~ 25.0% in 890 T_2 seeds, whereas 18% in the wild type. Of the 890 T_2 seeds, 453 T_2 seeds showed lower apparent amylose content and the remaining 197 T_2 seeds were higher than 20%. This result had been observed in previous reports (Chen 2003; Wu et al. 2006). Indeed, the amylose content in T_1 seeds with sense expression of rice starch branching enzyme 1 gene was reduced, while the amylose content in T_1 seeds with antisense expression of rice starch branching enzyme 1 gene was increased compared with that of wild type (Wu et al. 2006). The amylose content in some T_1 seeds from *japonica* rice with antisense expression of rice *OsSbe1* gene had no significant change. However, the amylose content in T_1 seeds from *indica* rice with antisense expression of rice *OsSbe1* was reduced compared with that of wild type. Moreover, the amylose content in T_2 seeds was higher than that in T_1 seeds (Chen 2003).

The palatability from Toyo taste meter is positively and significantly correlated with palatability from sensory test, and thus the palatability from Toyo taste meter is considered an important and direct index in the evaluation of rice eating quality (Tanaka et al. 1992; Lestari et al. 2009). In this research, the palatability of 52 T_3 lines was 61.1 ~ 72.6, and the variability was 4.33% lesser than 10.90% of 22 japonica rice variteties (Lestari et al. 2009). This indicates that the palatability is a variant trait, and the high eating quality rice varieties could be developed. Shu et al. (1998) revealed that the breakdown viscosity of well known good quality rice varieties was higher than 100 RVU (rapid visco unit), while the setback viscosity was below 25 RVU, and tended to be negative in most cases. Contrarily, the breakdown viscosity of inferior quality rice varieties was always lower than 35 RVU, while the setback viscosity was higher than 80 RVU. The texture of cooked rice was proved to be closely related with RVA (Rapid Visco Analyzer) profile characters. The hardiness of cooked rice was significantly and positively correlated with setback viscosity, but negatively correlated with breakdown viscosity. In an opposite way, the adhesiveness of cooked rice was significantly and negatively correlated with setback viscosity and consistency viscosity. In this study, the breakdown viscosity of 52 T_3 lines was 94.2 ~ 170.4 RVU, while 112.6 RVU in the wild type. Moreover, the setback viscosity varied from -60.0 to 22.5 RVU, while -6.17 RVU in the wild type. These results were consistent with the reported of Shu et al. (1998).

The transgene copy number does not have an inevitable correlation with target trait improvement. In this study, the wide range of palatability in T_3 lines was not due to differences in the number of transgene copy, as transgenic lines with the same transgene copies showed different eating quality. The transgene copies were not significantly correlated with rice eating quality (Table 5). In fact, Aluru et al. (2008) reported that the variability of carotenoid content was not due to differences in the numbers of transgenes, as both high and low β-carotene lines had similar numbers of *crtB* and *crtI* genes. The variability in the copy number could be due to the positional effects, germplasm used for transformation, and epigenetic effect. Overepxression of *OsSbe1* showed no significant alteration in the flowering date and panicle length. However, the eating quality, milled rice yield, panicle fertility, 1000-grain weight, spikelets per panicle, panicles per plant, and panicle length displayed difference between wild type and transgenic line groups. Additionally, mRNA expressions of *OsSbe1* at 10 and 15 days after flowering seeds were increased in the high eating quality lines compared with wild type. Moreover, starch branching enzyme activity at 25 days after flowering seeds in high eating quality lines was higher than that in wild type, in which starch branching enzyme activity was positively and significantly correlated with eating quality in *japonica* rice (Table 5). Thus, the overexpression of *OsSbe1* gene in a *japonica* rice variety has considerable potential for improving the eating quality and milled rice yield in *japonica* rice. The high eating quality transgenic rice lines can be used as a donor in breeding programs,

and lead to the creation of varieties that are fully integrated with the demands of modern rice-eating consumers.

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