Research Paper

A single amino acid mutation of *OsSBEIIb* contributes to resistant starch accumulation in rice

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Foods rich in resistant starch can help prevent various diseases, including diabetes, colon cancers, diarrhea, and chronic renal and hepatic diseases. Variations in starch biosynthesis enzymes could contribute to the high content of resistant starch in some cultivars of rice (*Oryza sativa* L.). Our previously published work indicated that the *sbe3-rs* gene in the rice mutant line, 'Jiangtangdao1' was a putative allele of the rice starch branching enzyme gene *SBEIIb* (previously known as *SBE3*); *sbe3-rs* might control the biosynthesis of the high resistant starch content in the rice line. Biomolecular analysis showed that the activity of SBEs was significantly lower in soluble extracts of immature seeds harvested from 'Jiangtangdao1' 15 days after flowering than in the extracts of the wild-type rice line 'Huaqingdao'. We performed gene complementation assays by introducing the wild-type *OsSBEIIb* into the *sbe3-rs* mutant 'Jiangtangdao1'. The genetically complemented lines demonstrated restored seed-related traits. The structures of endosperm amylopectin and the morphological and physicochemical properties of the starch granules in the transformants recovered to wild-type levels. This study provides evidence that *sbe3-rs* is a novel allele of *OsSBEIIb*, responsible for biosynthesis of high resistant starch in 'Jiangtangdao1'.

Key Words: resistant starch, *sbe3-rs*, rice, starch branching enzyme, complementation assay, Jiangtangdao1.

Introduction

Starch is a biologically and commercially important polymer of glucose synthesized and stored in the form of starch grains inside amyloplasts. Most of the starch in cereal products is digested rapidly in the upper gastrointestinal tract and is called "rapidly digested starch" (RDS). However, a proportion of the starch in cereals is resistant to enzymatic hydrolysis and hence escapes degradation in the stomach and the small intestine; this starch is termed "resistant starch" (RS; Englyst *et al.* 1982). RS is recognized as a significant contributor to gastrointestinal health. It is fermented by microorganisms in a large bowel to produce short chain fatty acids (SCFAs) including acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate, which are thought to promote the optimal function of the viscera (Perera *et al.* 2010, Topping and Clifton 2001, Wong *et al.* 2006). RS can

be classified into the following four groups: RS1, RS2, RS3, and RS4 (Perera *et al.* 2010). RS is considered to be beneficial for both prevention and management of diet-related illnesses, such as type-II diabetes, obesity, and colorectal cancer (Wolever 2003). RS has been paid attracting attention from geneticists and breeders for the last two decades. Many high-amylose cultivars of rice (*Oryza sativa*), maize (*Zea mays*), and barley (*Hordeum vulgare*) developed by mutation or biotechnological techniques have been found to contain high RS content (Bird *et al.* 2008, Hallström *et al.* 2011, Jiang *et al.* 2010).

Starch biosynthesis is a complicated process in plants. Three groups of enzymes are known to be involved in the synthesis pathways (Hennen-Bierwagen *et al.* 2008). There are starch synthases (SSs), which catalyze the formation of linear α-1,4 linked glucan chains, disbranching enzymes (DBEs) and pullulanase, which are important for the formation of semi-crystalline amylopectin (Fujita *et al.* 2009, Wattebled *et al.* 2005), and starch branching enzymes (SBEs), which play an essential role in starch biosynthesis, catalyzing chain transfer by cleavage of an α-1,4 linkage following the condensation of an α-1,6 linkage (Tanaka *et al.* 2004). SBE isoenzymes have been characterized in

many important crops, such as potato tuber (*Solanum tuberosum*; Larsson *et al.* 1996), rice (Mizuno *et al.* 1993), maize (Boyer and Preiss 1978), barley (Sun *et al.* 1997), and wheat (Morell *et al.* 1997). There are two different classes of SBEs in cereals: SBEI and SBEII (which is further classified into SBEIIa, and SBEIIb). Rice has three SBE isoforms, SBEI, SBEIIa (previously known as SBE4), and SBEIIb (previously known as SBE3). SBEI plays a role in the formation of long chains of amylopectin, producing a range of short and intermediate chains with degree of polymerization (DP) ≤ 40. SBEIIb generates short chains; *in vitro* experiments with rice enzymes revealed that SBEIIb transferred chains of DP6 and DP7. Additionally, SBEIIa transferred chains of DP6–15, which partially, but not fully, supports the functions of SBEI and IIb (Zhu *et al.* 2012). Alterations in SBE activity affect the number and size distribution of amylopectin branches. Loss of activity of SBEI in maize endosperm, rice endosperm, or potato tuber did not alter starch content and led to minor differences in amylopectin structure and/or the properties of starch (Blauth *et al.* 2002, Satoh *et al.* 2003, Xia *et al.* 2011). In contrast, loss of SBEII resulted in significant changes, such as an increase in the apparent amylose content (AAC), as observed in several species, including maize (Stinard *et al.* 1993), potato (Jobling *et al.* 1999), pea (Bhattacharyya *et al.* 1990), rice (Mizuno *et al.* 1993), barley (Regina *et al.* 2010), and wheat (Regina *et al.* 2006).

In rice, allelic variations in genes related to starch synthesis affect phenotypic variations in grain quality traits (Mo *et al.* 2014). Mutations in *SBEIIb* and the resulting phenotypes have been reported in 'japonica' and 'indica' backgrounds. For example, an SNP causing a G/C transversion at the downstream end of the *SBEIIb* gene was significantly associated with multiple trait indices (Lu and Park 2012). In a previous study, we located a putative gene, *sbe3-rs*, in mutant 'Jiangtangdao1', which contained a missense mutation, Leu-599-Pro, in *SBEIIb* (Yang *et al.* 2012). This locus explained 60.4% of the RS variation in the F_2 population of a cross between 'Jiangtangdao1' and 'Milyang23'.

To further determine whether or not the high RS content in 'Jiangtangdao1' was caused by *sbe3-rs*, in the present study, we conducted a genetic complementation of the *sbe3-rs* gene in 'Jiangtangdao1' with the wild-type copy of *SBEIIb* using transgenic technology.

Materials and Methods

Plant materials

This study was conducted using the rice line 'Jiangtangdao1', which is a high-RS rice mutant from a double haploid (DH) population, derived from 'Huaqingdao' by treating its young panicles with 0.015% N-methylnitrosourea (NMU; Yang *et al.* 2012). The original parent 'Huaqingdao' was used as a wild-type control. The seeds of 'Jiangtangdao1' and 'Huaqingdao' were harvested at 15 days after flowering (DAF), immediately frozen in liquid nitrogen,

and stored at –80°C until use for analysis of gene expression and enzyme activity.

Gene expression analysis by RNA-seq and quantitative RT-PCR

For RNA-seq, total RNA was extracted from seeds of 'Huaqingdao' and 'Jiangtagndao1' 15 DAF using a Quick RNA isolation kit (Biotech Corporation, Beijing, China), according to the manufacturer's protocol. RNA-seq analysis was performed on an Illumina High-Seq 2500 platform by a commercial service provider (Shanghai Hanyu Bio-Tech, China). Raw sequences in FASTQ format obtained from the Illumina platform were analyzed using freely available tools (http://hannonlab.cshl.edu/fastx_toolkit/). The sequences were mapped to the rice reference genome sequence using several programs. The expression level of each transcript was expressed as the fragments per transcript kilobase per million fragments mapped value, which was calculated based on the number of mapped reads. Log2 transformation was performed on the reads per kilobases per million reads (RPKM) data to facilitate graphical comparison of the expression of the genes (Fernandez-Aparicio *et al.* 2013). Quantitative real-time PCR (qRT-PCR) was performed in a QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using 100 ng of cDNA template for amplification. The primers using for the branching enzyme were SBEIIb-F (5′-GACGACTTGCTGT CCTCT-3′) and SBEIIb-R (5′-CAACCTTTATGCTTGCTT-3′). Rice Actin1 cDNA, amplified with the primers ActinF (5′-CATCGTTCTCAGTGGTGG-3′) and ActinR (5′-CAT CTGCTGGAATGTGCT-3′), was used as a reference. Real-time PCR amplification was conducted using SYBR Green I PCR Master Mix Kit (TaKaRa). Relative expression levels were calculated using the ΔΔCT method (Livak and Schmittgen 2001).

Determination of starch branching enzyme activities

Starch branching enzyme (SBE) activity was quantified as described in a previous report (Cheng *et al.* 2001). The panicles were de-hulled manually, and the fresh weight of 20 grains was recorded. The enzymes were extracted and analyzed according to the methods described by Cheng *et al.* (2001). The optical density of the final product was measured at 540 nm. The experiments were performed on ice in triplicate.

Cloning and sequencing of the SBEIIb gene and its promoter

Total RNA was isolated from seeds (15 DAF) of 'Huaqingdao' using an RNA extraction kit (SK8661; Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. First-strand cDNA synthesis of rice genes was carried out using a cDNA synthesis kit (TaKaRa, Japan). The complete coding sequence of *SBEIIb* was amplified from these cDNAs using gene-specific primers containing *Sal*I and *Xba*I restriction sites (upstream primer: 5′-GATCGTCGACTGAGGAGGGTTTAGGTGGAAG-3′;

downstream primer: 5′-GTACTCTAGAGCCTCTTGGTGT TCTCATTCC-3′). The amplified cDNA products were cloned into the pMD18-T vector (TaKaRa). The resulting plasmid, pMD18-SBEIIb, containing a 2563-bp cDNA fragment including the 2478-bp *SBEIIb* coding region, was verified by sequencing. The 2.254-Kb *SBEIIb* promoter was obtained via polymerase chain reaction (PCR) using the following primers containing *Hind*III and *Sal*I sites: 5′-GATCAAGCTTCAGCAAGTGACGGTGTTCG-3′ and 5′-GTACGTCGACTTCCACCTAAACCCTCCTCA-3′. Thereafter, the promoter was cloned into a pMD18-T vector (TaKaRa) to create the pMD18-T-SBEP plasmid. The resulting plasmid was sequenced and analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The correct promoter was digested with *Hind*III and *Sal*I and ligated into an expression plasmid, p1300nos (constructed by inserting a Nos terminator in the multiple cloning site of pCAMBIA1300) to create p1300-SBEp-nos. Finally, the plasmid pMD18-SBEIIb was digested with *Sal*I and *Xba*I and ligated into p1300-SBEp-nos to create the binary vector p1300-SBEp-SBE-nos.

Transformation of the high-RS mutant, 'Jiangtangdao1', and screening of transgenic lines

The procedures for rice tissue culture and transformation with *Agrobacterium tumefaciens* were as described by Yang *et al.* (2011). The plasmid p1300-SBEp-SBE-nos was introduced into the *A. tumefaciens* strain EHA105, which was used to transform the rice embryogenic callus cultured from 'Jiangtangdao1'. The weight of the T_1 generation seeds from three T_0 lines (No. 1, 19, and 20) and 'Huaqingdao' was examined; seed yield-related traits of the transgenic line were evaluated for 10 plants of each line. The host 'Jiangtangdao1' and the wild-type 'Huaqingdao' plants were used as controls.

DNA extraction and transgene copy number analysis by quantitative real-time PCR

The plant genomic DNA samples used for qPCR were extracted and purified using a genomic DNA extraction kit (Axygen, Union City, CA, USA). Genomic DNA was quantified by determining the absorbance at 260/280 nm and stored at −20°C until further analysis. The oligonucleotide primers were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). All primers were synthesized by Shanghai Sangon Biotech (China). The primers for the endogenous gene *SPS* were: SPSF (5′-TTG CGCCTGAACGGATAT-3′) and SPSR (5′-CGGTTGATCT TTTCGGGATG-3′). The primers for target gene *HPT* were HPTF (5′-AACTCCCCAATGTCAAGC-3′) and HPTR (5′-GTCCTGCGGGTAAATAGC-3′). qPCR was performed in a QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems). The reaction mixtures containing 2 μL of DNA and 18 μL of PCR master mixture were transferred to 96-well plates for amplification. The PCR master mixture contained 10 μL of 2X TaKaRa SYBR Premix Ex Taq™

(RR041, TaKaRa, Tokyo, Japan), 0.4 μL of ROX Reference Dye (50X), 0.4 μL of each primer (10 mM), and 6.8 μL of ddH$_2$O. Reactions were performed at 95°C for 1 min (1 cycle), followed by 95°C for 15 s and 60°C for 30 s (40 cycles). Each reaction was performed using three replicates and was repeated three times. The *SPS* gene, which is a single copy gene in the rice genome, was used as the endogenous reference gene. We used a real-time quantitative PCR method to calculate the *HPT* copy number according to the methods of Yang *et al.* (2005).

Measurement of apparent amylose content and RS content

RS content was measured using the Megazyme RS assay kit (Megazyme, Co. Wicklow, Ireland) according to manufacturer's instructions; this kit has been widely used for RS determination in crops (McCleary *et al.* 2002). The apparent amylose content (AAC) of starch was measured using a simplified I$_2$/KI assay as described by Yang *et al.* (2006). Four standard samples, each with a different AAC (1.2%, 11.2%, 16.8% and 26.8%), were procured from the China National Rice Research Institute (CNRRI, China). The experiments were performed in triplicate.

Scanning electron microscopy (SEM)

Brown (unpolished) rice was broken manually and cut into sections approximately 2 mm thin. After coating with Pt ions in an argon atmosphere for 25 min (using an IB-5 Ion coater, Eiko Engineering, Ibaraki, Japan), the cross-sections were adhered to a double-adhesive tape that was fixed to a metallic stud. Starch granules in the sections were visualized using an electron microscope (FEI NovaNano SEM430, FEI Co., Hislsboro, OR, USA).

Determination of the chain length distribution of disbranched α-glucans

The chain length distribution of α-glucans in the transformants, 'Jiangtangdao1' and 'Huaqingdao', was determined according to the capillary electrophoresis method (O'Shea and Morell 1996). The measurements were conducted using a BioLC system (model DX-500, Dionex, Sunnyvale, CA, USA).

X-ray diffraction analysis of starch

These procedures were performed according to the methods described by Sawada *et al.* (2009).

Results

Gene expression analyses and detection of branching enzyme activity

Our previously published work indicated that *sbe3-rs* might control the biosynthesis of RS and its accumulation to high levels in the 'Jiangtangdao1' rice line. To assess the expression of SBE genes (*SBEI*, *SBEIIa*, and *SBEIIb*) in immature rice seeds of the wild type and the mutant, RNA-seq and qPCR analyses were performed. The expression
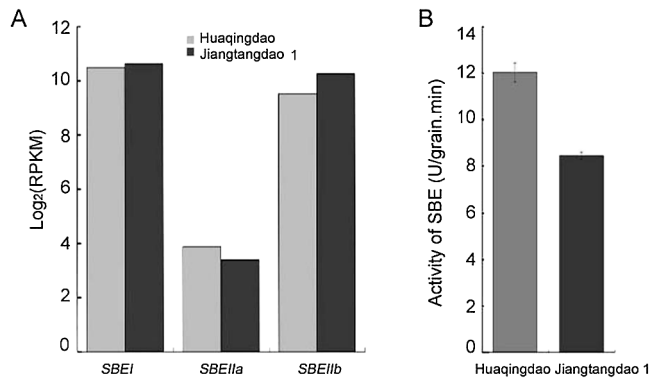
Fig. 1. Expression analyses and activity detection of starch branching enzyme (SBE). A. Transcriptional levels of three *SBE* isoforms determined as RPKM values based on RNA-seq analysis and presented as log2. B. Activities of SBE in immature seeds of 'Jiangtangdao1' and 'Huaqingdao'.

level of each transcript was denoted in terms of the FPKM value. The RPKM values for the three genes are presented in **Fig. 1A**. The RNA-seq results revealed that the expression levels of the three SBE isoforms in 'Jiangtangdao1' were not significantly different from those in 'Huaqingdao'. *SBEIIb* transcript levels in 'Jiangtangdao1' and control plants were checked by qRT-PCR. The results revealed that the level of *SBEIIb* in the immature seeds of the wild-type 'Huaqingdao' was 1.6 times higher than in 'Jiangtangdao1' seeds. There were no significant differences in the *SBEIIb* expression levels between the mutant and the wild type.

The activity of SBEs was measured in the soluble extracts of immature seeds harvested 15 days after flowering (**Fig. 1B**). The activity of SBEI, SBEIIa, and SBEIIb was evaluated. The specific activity in the mutant 'Jiangtangdao1' was approximately 70% of that in the wild-type 'Huaqingdao'. This result was similar to that of amylose-extender mutants. The activity of the branching enzyme in the rice amylose-extender mutants was approximately 60–90% of that in the wild-type plants (Mizuno *et al.* 1993). Thus, these results indicated that the high content of RS in 'Jiangtangdao1' likely to have resulted from reduced activity of *SBEIIb* and was not a consequence of the differential expression of the gene.

Generation of transgenic plants with a single copy number of the transgene

To complement *sbe3-rs* in 'Jiangtangdao1', a native *SBEIIb* promoter-driven *SBEIIb* gene expression cassette was constructed (**Fig. 2A**). The wild-type *SBEIIb* coding sequence and *SBEIIb* promoter of 'Huaqingdao' were amplified by PCR and inserted into a binary plasmid. Transgenic rice plants were produced by introducing the construct into 'Jiangtangdao1'. In total, 50 independent T_0 transgenic lines were obtained and grown in a glasshouse maintained at 28°C during the day (12 h) and 24°C during the night (12 h). We employed a simple and effective quan-

titative method to estimate the transgene copy number in the transgenic rice. In this approach, the copy number was determined relative to an endogenous reference gene, *SPS*, which is a single-copy gene in rice (Ding *et al.* 2004, Yang *et al.* 2005). The analysis revealed the presence of a single transgene copy in 21 independent T_0 lines. Of these, three independent T_0 complemented lines (T_0-1, 19, and 20) were chosen for further examination.

OsSBEIIb complemented sbe3-rs phenotypes in Jiangtangdao1

We examined the phenotypic changes in three individual T_0 lines. The dry weight of dehulled seeds of 'Jiangtangdao1' was significantly lower (19.8 ± 1.7 mg) than that of the wild-type cultivar, 'Huaqingdao' (26.3 ± 0.9 mg). The dry weight of mature T_1 seeds of the three transgenic lines (T_0-1, 19, and 20) was significantly higher than that of the T_1 seeds of 'Jiangtangdao1' (**Fig. 2B**). Twelve randomly chosen T_1 seeds of T_0-1 plants were grown in the greenhouse, and T_2 seeds obtained from them were further examined for their phenotypes. The homozygous phenotype was inherited in the seeds of plant No. 1–5 in the following generation; biochemical and morphological analyses were performed using the maturing T_3 seeds of these plants. The unpolished grains of the high-RS mutant, 'Jiangtangdao1', had an almost opaque endosperm because of increased chalkiness (**Fig. 2C**). In contrast, the grains of 'Huaqingdao' were quite transparent (**Fig. 2C**). However, all the seeds of the No. 1–5 transgenic plants reverted to the transparent endosperm phenotype, which was similar to the wild type (**Fig. 3C**).

The content of amylose and RS in rice grains was measured. 'Jiangtangdao1' had high RS (11.67 ± 0.43%) and amylose (31.1 ± 0.15%) content, whereas 'Huaqingdao' had low RS (0.38 ± 0.12%) and amylose (16.27 ± 0.21%) content. The RS (1.21 ± 0.14%) and amylose contents (19.19 ± 0.19%) in the complemented lines (No. 1–5) were significantly lower than those in 'Jiangtangdao1' and almost equal to those in the wild type.

The effects of the *SBEIIb* gene on several traits related to seed yield were further evaluated in plants of the T_3 generation obtained from the complemented lines 1–5. No marked changes were observed in the number of panicles per plant, number of grains per panicle, or rate of seed-set. However, the thousand-seed weight was significantly increased in the transgenic plants (**Table 1**).

Recovery of morphological properties of the starch granules in the complemented lines

As the endosperm chalkiness was higher in 'Jiangtangdao1', we speculated that the morphological properties of the starch granules in the endosperm could have changed. To test the morphological differences of starch granules among 'Jiangtangdao1' plants, the transformants, and the wild-type 'Huaqingdao' plants, we used SEM imaging (**Fig. 3**). The starch granules of the high-RS mutant 'Jiangtangdao1' were
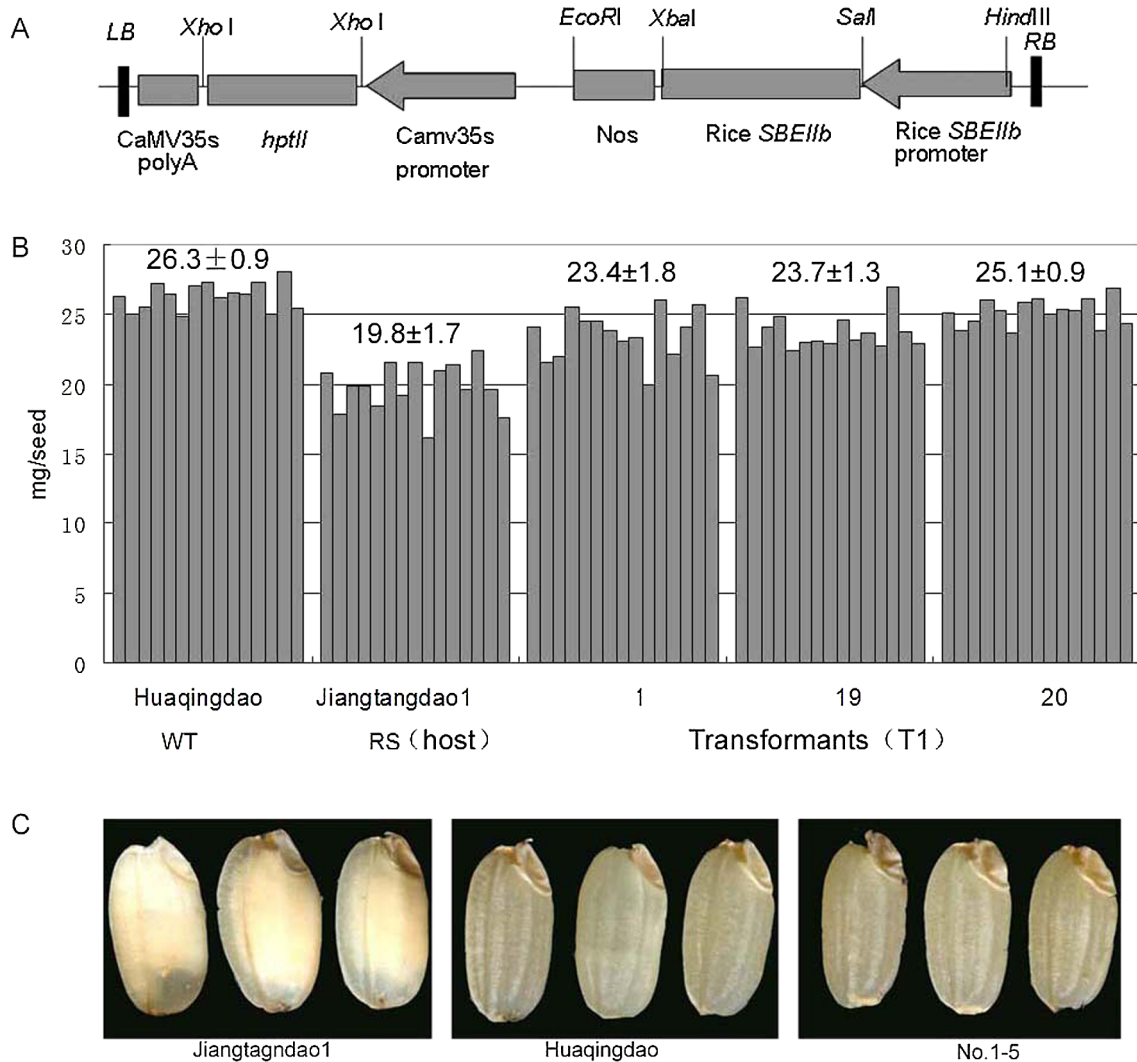
Fig. 2. Transformation of high-RS mutant rice line 'Jiangtangdao1' with rice wild-type *SBEIIb* gene. A. Construct with the *OsSBEIIb* gene used for transformation of 'Jiangtagndao1'. The clone contained the promoter region up to 2.3 kb upstream from the transcription start site and the transcribed region for the *OsSBEIIb* gene. B. Weight of individual mature T_1 kernels of the three transformed rice lines No-1, 19, and 20, the host mutant line 'Jiangtangdao1' and the wild-type cultivar 'Huaqingdao'. The numbers above the bars indicate the average values (mg) ± SD (mg) (n = 15). C. Endosperm of 'Jiangtangdao1', 'Huaqingdao' and T_3 seeds of the transformed line No. 1–5, respectively.

large, non-angular, and rounded; some were elongated, whereas others were small and polygonal (**Fig. 3A**). Starch granules in the wild-type 'Huaqingdao' mainly consisted of similar-sized polygonal granules with sharp angles and edges. The granules of 'Jiangtangdao1' were much more variable in size than the polygonal granules of the wild-type 'Huaqingdao'. However, the shape of starch granules of *OsSBEIIb* transformants approached those of the wild-type plants, except for the size of the starch granules, which were slightly smaller than the granules in the wild type (**Fig. 3B**, **3C**). This result demonstrated the chalky properties of *sbe3-rs* mutant were fully complemented by the introduction of wild-type copy of *OsSBEIIb*.

Distribution of chain length in amylopectin and X-ray diffraction pattern of the starch granules in the 'Jiangtangdao1' transformants

As *SBEIIb* played a specific role in the formation of branches in the crystalline lamellae of the amylopectin cluster in rice endosperm, it affected the distribution of chain length in amylopectin (Nishi *et al.* 2001). **Fig. 4** shows that in the high-RS-containing 'Jiangtangdao1' rice, the proportion of short chains (DP ≤ 13) was apparently lower than that in the wild-type 'Huaqingdao'. In contrast, the proportion of longer chains in the cluster linking of long chains (DP ≥ 15) was increased considerably (**Fig. 4A**). The amylopectin chain length distribution profile of *OsSBEIIb* transformants was very similar to that of the wild type (**Fig. 4B**).

As revealed by X-ray powder diffraction, the starch granule crystals exhibited three different structures designated as
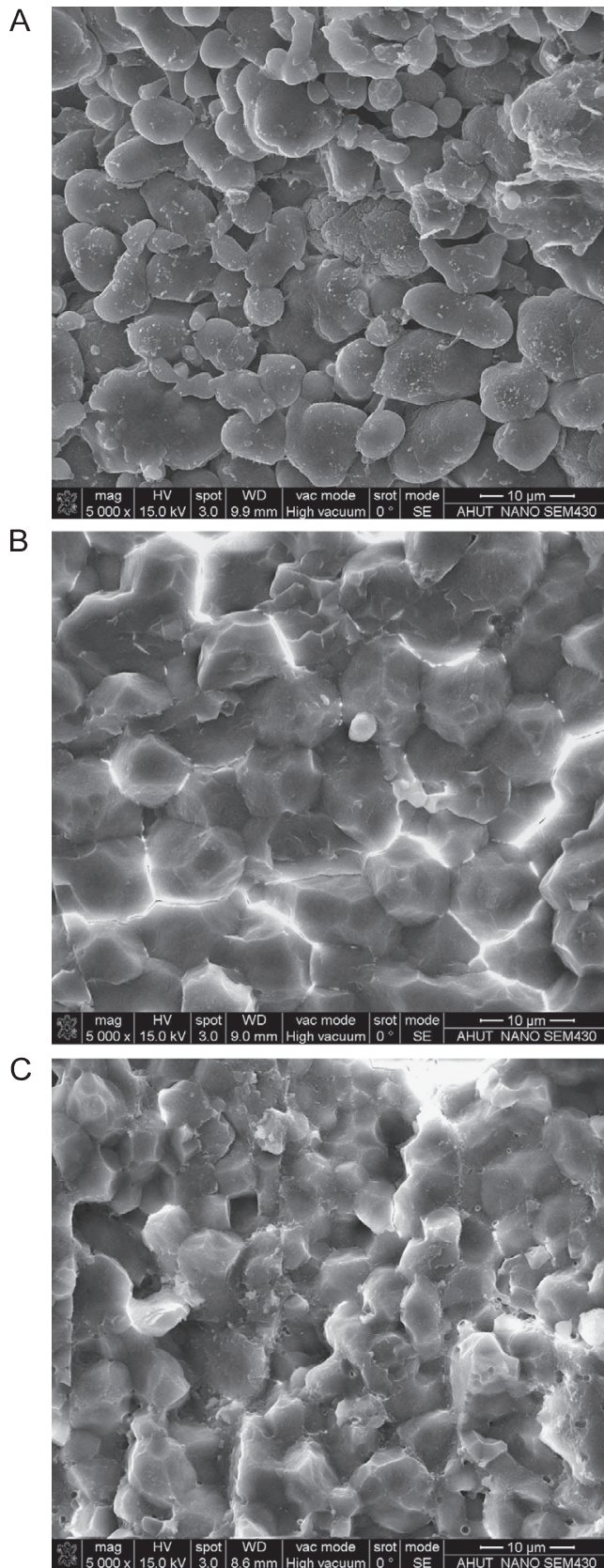
A

B

C

Fig. 3. Scanning electron micrographs of starch granules in endosperm. A. 'Jiangtangdao1'. B. 'Huaqingdao'. C. Transgenic lines No. 1–5.

A-, B-, and C-type allomorphs. The C-type allomorph was a mixture of both A- and B-type allomorphs. Previous reports have indicated that the starch granules of rice amylose-extender mutants have a B-type X-ray diffraction pattern, whereas those of the wild-type show a typical A-type pattern (Nishi *et al.* 2001). As shown in **Fig. 5**, the endosperm starch of 'Huaqingdao' had the A-type X-ray diffraction pattern, in contrast to the B-type diffraction pattern observed in the mutant 'Jiangtangdao1'. The typical B-type diffraction pattern of the starch granules in 'Jiangtangdao1' was changed to a typical A-type pattern in the transformants.

All of these results indicated that *OsSBEIIb* could complement the *sbe3-rs* in the high-RS-content line, 'Jiangtangdao1'.

Discussion

In cereals, *SBEIIb* plays a specific role in the formation of short chains within the amylopectin cluster during starch synthesis (Nishi *et al.* 2001). It should be noted that in this study, the effect of *SBEIIb* on the structure of amylopectin was entirely dependent on its activity. Many researchers have reported that down-regulation of *SBEII* could lead to high amylose content in mutant maize, wheat, barley, and rice (Butardo *et al.* 2011, Wei *et al.* 2010, Zhao *et al.* 2015). The inactivation of *SBEIIb* in rice is traditionally associated with elevated AAC, increased gelatinization temperature, and a decreased proportion of short amylopectin branches (Butardo *et al.* 2011, Nishi *et al.* 2001). In general, the RS content of granular starch is positively correlated with the level of amylose (Sang *et al.* 2008). High RS always results from high amylose content (Perera *et al.* 2010). The mutant 'Jiangtangdao1' was identified from a double haploid (DH) population derived from 'Huaqingdao'. The RS content was significantly increased from $0.38 \pm 0.12\%$ in the wild-type 'Huaqingdao' to $11.67 \pm 0.43\%$ in 'Jiangtangdao1'. Using an F_2 population, we previously mapped *sbe3-rs* to *SBEIIb*, a recessive allele for high RS in 'Jiangtangdao1' (Yang *et al.* 2012). Previous studies showed that the activity of SBE was highest from 13 DAF to 15 DAF (Cheng *et al.* 2001, Shu *et al.* 2014). Therefore, in the present study, gene expression in the mutant 'Jiangtangdao1' and the wild-type 'Huaqingdao' was analyzed 13–15 DAF by RNA-seq. No differential expression of the three rice *SBE* isoforms (*SBEI*, *SBEIIa*, and *SBEIIb*) was observed. The nucleotide sequence is the basis of gene expression and gene function, and single-nucleotide polymorphism (SNP) and insertions or deletions in genes related to amylopectin structure among different rice varieties have been reported. In a previous study, a G-A SNP was identified in a high-AAC rice mutant, IR36ae, which resulted in an improper stop codon that could potentially lead to the formation of a truncated *SBEIIb* (Butardo *et al.* 2012). Although the structure of starch and the resulting functional properties of 'Jiangtangdao1' were consistent with those of IR36ae, a T-C mutation was found in 'Jiangtangdao1' (Yang

Table 1. Traits related to seed yield in transformants, host 'Jiangtangdao1', and the control 'Huaqingdao' (Shanghai, China, 2015)

Line	Panicle length (cm)	Panicles per plant	Grains per panicle	Seed-setting rate (%)	Thousand-seed weight (g)
Huaqingdao (CK)	16.4 ± 1.3	11.2 ± 1.2	121.6 ± 23.2	94.2 ± 2.5	24.4 ± 1.1
Jiangtangdao1	16.7 ± 1.8	12.3 ± 0.9	112.1 ± 19.3	91.1 ± 2.9	19.9 ± 2.1*
No. 1–5	16.2 ± 1.2	10.9 ± 1.1	115.1 ± 20.2	92.7 ± 2.4	22.3 ± 1.8

Note: 10 plants from the transformed and the control lines were evaluated and the measurements were taken when the plants were mature.
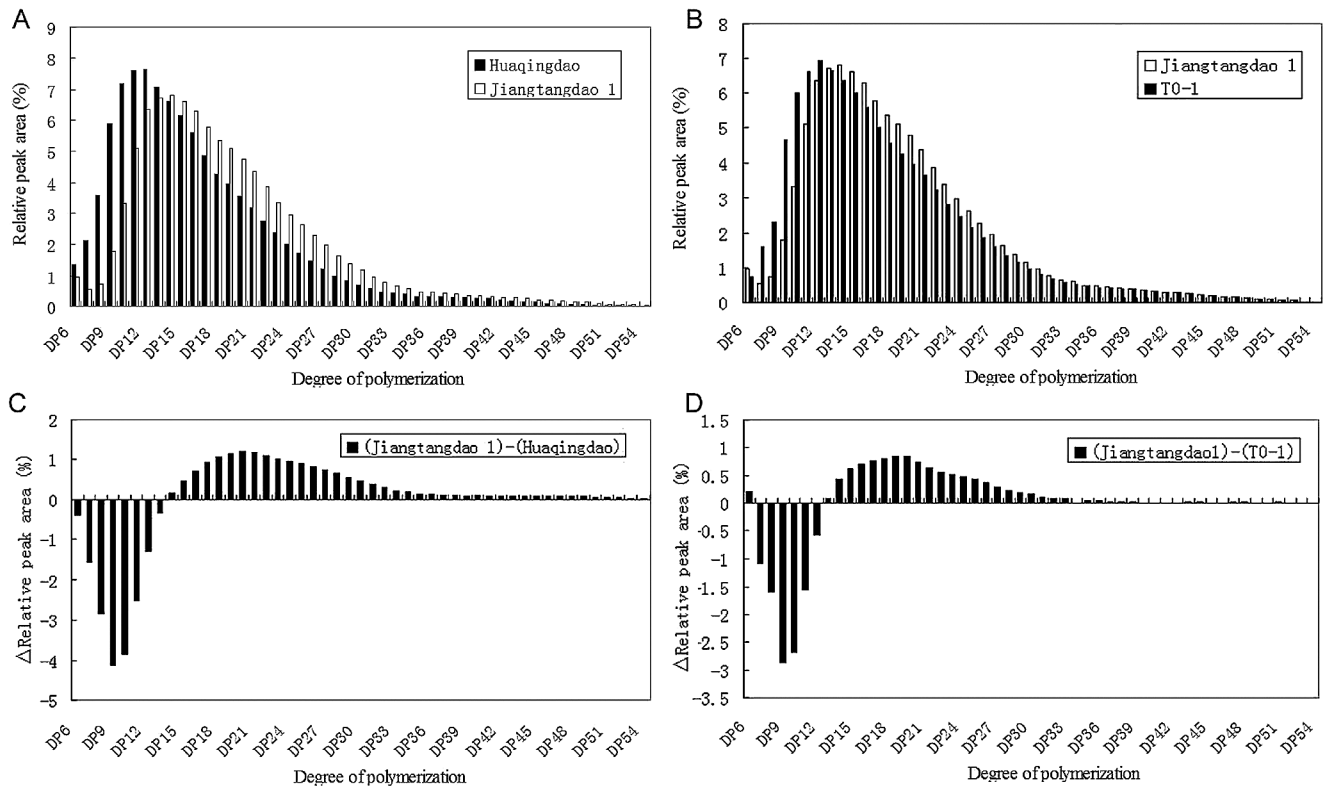* Significant at the 0.05 probability level.

Fig. 4. Comparison of chain length distribution of amylopectin in mature endosperm in the transformed rice, its high-RS-containing host 'Jiangtangdao1', and the wild-type cultivar 'Huaqingdao'.
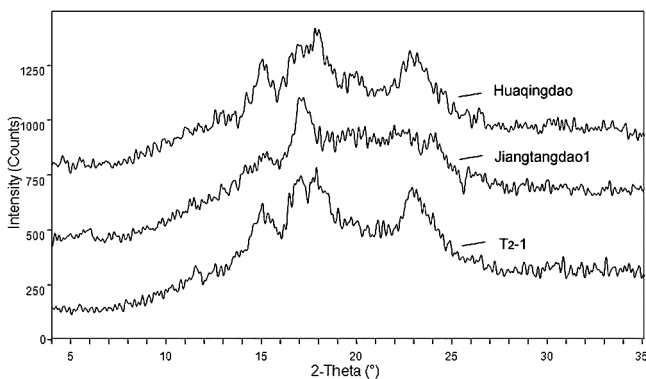

Fig. 5. X-ray diffraction patterns of purified starch granules in the mature endosperm of the rice transformant, its mutant host 'Jiangtangdao1', and the wild-type cultivar 'Huaqingdao'.

et al. 2012) that did not affect the expression of *SBEIIb*. This indicated that the mechanism of high RS and AAC accumulation in 'Jiangtangdao1' was different from that previously reported for IR36ae.

High-amylose mutants (amylose-extender) of rice are characterized by higher AAC, decreased levels of short chains of amylopectin, higher gelatinization temperature, B-type X-ray diffraction, and a particular morphology of starch granules (Yano *et al.* 1985). 'Jiangtangdao1' was similar to rice amylose-extender mutants with high AAC and B-type X-ray diffraction but for its high RS content. An earlier study demonstrated that branching enzyme activity was significantly decreased in the amylose-extender mutants (Mizuno *et al.* 1993, Nishi *et al.* 2001). Our results were consistent with these previously reported findings (**Fig. 1B**). The level of the specific activity in the mutant

'Jiangtangdao1' was approximately 70% of that in the wild-type 'Huaqingdao', which indicated that the mutation in *SBEIIb* (leading to the replacement of Leu with Pro) might have affected the SBE activity, thereby resulting in the high AAC and RS content in 'Jiangtangdao1'.

We further performed functional complementation of the *sbe3-rs* gene in 'Jiangtangdao1' with wild-type *SBEIIb*. Wild-type rice *SBEIIb* was introduced into the high-RS mutant 'Jiangtangdao1'. The introduction of the gene had no physiological effects on the vegetative tissues of the transgenic plants because *SBEIIb* was specifically expressed in the endosperm of rice and its expression was driven by its own promoter. When *SBEIIb* was expressed in the 'Jiangtangdao1' mutant, the RS content of 'Jiangtangdao1' (11.67%) was reduced to 1.21%, thus indicating that *sbe3-rs* was an allele of *SBEIIb*. The *ae* mutants in cereals are often associated with slight reductions in grain weight; this was also observed in 'Jiangtangdao1' (**Fig. 2**). The dry weight of grain in 'Jiangtangdao1' was significantly lower (19.8 ± 1.7 mg) than in the wild-type 'Huaqingdao' (26.3 ± 0.9 mg). However, the seed weight in the transformants was similar to that in the wild type. The structure of amylopectin from the mutant Jiangtangdao1 was clearly distinct from that of the amylopectin of the wild-type plants. In 'Jiangtangdao1', the proportion of short chains with DP ≤ 13 was markedly lower and the proportion of long chains of DP = 13–30 and DP ≥ 40 was higher than that observed in the wild type (**Fig. 4**); this was consistent with previous observations (Butardo *et al.* 2011, Nishi *et al.* 2001). The fine structure of amylopectin in the transformants of 'Jiangtangdao1' exhibited great variations (**Figs. 4, 5**).

In conclusion, the present study indicated that the variation in SBEIIb enzyme activity probably caused the different phenotypes of 'Jiangtangdao1' and that the wild-type rice *SBEIIb* gene could complement the mutant *sbe3-rs* gene in Jiangtangdao1. The high RS content in 'Jiangtagndao1' was, in fact, caused by a missense mutation that changed Leu599 to Pro599. Further molecular genetic and biochemical analyses of this mutation are required to fully elucidate the exact mechanism of high RS accumulation in Jiangtangdao1.

Literature Cited

Bhattacharyya, M.K., A.M. Smith, T.H. Ellis, C. Hedley and C. Martin (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell 60: 115–122.

Bird, A.R., M.S. Vuaran, R.A. King, M. Noakes, J. Keogh, M.K. Morell and D.L. Topping (2008) Wholegrain foods made from a novel high-amylose barley variety (*Himalaya 292*) improve indices of bowel health in human subjects. Br. J. Nutr. 99: 1032–1040.

Blauth, S.L., K.N. Kim, J. Klucinec, J.C. Shannon, D. Thompson and M. Guiltinan (2002) Identification of *Mutator* insertional mutants of starch-branching enzyme 1 (*sbe1*) in *Zea mays* L. Plant Mol. Biol. 48: 287–297.

Boyer, C.D. and J. Preiss (1978) Mutiple forms of (1→4)-α-D-gulcan, (1→4)-α-D-gulcan-6-glycosyl transferase from developing *zea mays* L. kernels. Carbohydr. Res. 61: 321–334.

Butardo, V.M., M.A. Fitzgerald, A.R. Bird, M.J. Gidley, B.M. Flanagan, O. Larroque, A.P. Resurreccion, H.K. Laidlaw, S.A. Jobling, M.K. Morell *et al.* (2011) Impact of down-regulation of *starch branching enzyme IIb* in rice by artificial microRNA- and hairpin RNA-mediated RNA silencing. J. Exp. Bot. 62: 4927–4941.

Butardo, V.M. Jr., V.D. Daygon, M.L. Colgrave, P.M. Campbell, A. Resurreccion, R.P. Cuevas, S.A. Jobling, I. Tetlow, S. Rahman, M. Morell *et al.* (2012) Biomolecular analyses of starch and starch granule proteins in the high-amylose rice mutant Goami 2. J. Agric. Food Chem. 60: 11576–11585.

Cheng, F.M., D.A. Jiang, P. Wu and C.H. Shi (2001) The dynamic change of starch synthesis enzymes during the grain filling stage and effects of temperature upon it. Acta Agron. Sin. 27: 201–206.

Ding, J., J. Jia, L. Yang, H. Wen, C. Zhang, W. Liu and D. Zhang (2004) Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. J. Agric. Food Chem. 52: 3372–3377.

Englyst, H., H.S. Wiggins and J.H. Cummings (1982) Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst 107: 307–318.

Fernandez-Aparicio, M., K. Huang, E.K. Wafula, L.A. Honaas, N.J. Wickett, M.P. Timko, C.W. Depamphilis, J.I. Yoder and J.H. Westwood (2013) Application of qRT-PCR and RNA-Seq analysis for the identification of housekeeping genes useful for normalization of gene expression values during *Striga hermonthica* development. Mol. Biol. Rep. 40: 3395–3407.

Fujita, N., Y. Toyosawa, Y. Utsumi, T. Higuchi, I. Hanashiro, A. Ikegami, S. Akuzawa, M. Yoshida, A. Mori, K. Inomata *et al.* (2009) Characterization of pullulanase (PUL)-deficient mutants of rice (*Oryza sativa* L.) and the function of PUL on starch biosynthesis in the developing rice endosperm. J. Exp. Bot. 60: 1009–1023.

Hallström, E., F. Sestili, D. Lafiandra, I. Björck and E. Östman (2011) A novel wheat variety with elevated content of amylose increases resistant starch formation and may beneficially influence glycaemia in healthy subjects. Food Nutr. Res. 55. doi: 10.3402/fnr.v55i0.7074.

Hennen-Bierwagen, T.A., F. Liu, R.S. Marsh, S. Kim, Q. Gan, I.J. Tetlow, M.J. Emes, M.G. James and A.M. Myers (2008) Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. Plant Physiol. 146: 1892–1908.

Jiang, H., J. Lio, M. Blanco, M. Campbell and J.L. Jane (2010) Resistant-starch formation in high-amylose maize starch during Kernel development. J. Agric. Food Chem. 58: 8043–8047.

Jobling, S.A., G.P. Schwall, R.J. Westcott, C.M. Sidebottom, M. Debet, M.J. Gidley, R. Jeffcoat and R. Safford (1999) A minor form of starch branching enzyme in potato (*Solanum tuberosum* L.) tubers has a major effect on starch structure: cloning and characterisation

of multiple forms of SBE A. Plant J. 18: 163–171.

Larsson, C.T., P. Hofvander, J. Khoshnoodi, B. Ek, L. Rask and H. Larsson (1996) Three isoforms of starch synthase and two isoforms of branching enzyme are present in potato tuber starch. Plant Sci. 117: 9–16.

Lu, F.H. and Y.J. Park (2012) An SNP downstream of the *OsBEIIb* gene is significantly associated with amylose content and viscosity properties in rice (*Oryza sativa* L.). J. Cereal Sci. 56: 706–712.

McCleary, B.V., M. McNally and P. Rossiter (2002) Measurement of resistant starch by enzymatic digestion in starch and selected plant materials: collaborative study. J AOAC Int. 85: 1103–1111.

Mizuno, K., T. Kawasaki, H. Shimada, H. Satoh, E. Kobayashi, S. Okumura, Y. Arai and T. Baba (1993) Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. J. Biol. Chem. 268: 19084–19091.

Mo, Y.J., J.U. Jeung, W.C. Shin, K.Y. Kim, C. Ye, E.D. Redona and B.K. Kim (2014) Effects of allelic variations in starch synthesis-related genes on grain quality traits of Korean nonglutinous rice varieties under different temperature conditions. Breed. Sci. 64: 164–175.

Morell, M.K., A. Blennow, B. Kosar-Hashemi and M.S. Samuel (1997) Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. Plant Physiol. 113: 201–208.

Nishi, A., Y. Nakamura, N. Tanaka and H. Satoh (2001) Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. Plant Physiol. 127: 459–472.

O'Shea, M.G. and M.K. Morell (1996) High resolution slab gel electrophoresis of 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) tagged oligosaccharides using a DNA sequencer. Electrophoresis 17: 681–686.

Perera, A., V. Meda and R.T. Tyler (2010) Resistant starch: A review of analytical protocols for determining resistant starch and of factors affecting the resistant starch content of foods. Food Res. Int. 43: 1959–1974.

Regina, A., A. Bird, D. Topping, S. Bowden, J. Freeman, T. Barsby, B. Kosar-Hashemi, Z. Li, S. Rahman and M. Morell (2006) High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc. Natl. Acad. Sci. USA 103: 3546–3551.

Regina, A., B. Kosar-Hashemi, S. Ling, Z. Li, S. Rahman and M. Morell (2010) Control of starch branching in barley defined through differential RNAi suppression of starch branching enzyme IIa and IIb. J. Exp. Bot. 61: 1469–1482.

Sang, Y.H., S. Bean, P.A. Seib, J. Pedersen and Y.C. Shi (2008) Structure and functional properties of sorghum starches differing in amylose content. J. Agric. Food Chem. 56: 6680–6685.

Satoh, H., A. Nishi, K. Yamashita, Y. Takemoto, Y. Tanaka, Y. Hosaka, A. Sakurai, N. Fujita and Y. Nakamura (2003) Starch-branching enzyme I-deficient mutation specifically affects the structure and properties of starch in rice endosperm. Plant Physiol. 133: 1111–1121.

Sawada, T., P.B. Francisco, Jr., S. Aihara, Y. Utsumi, M. Yoshida, Y. Oyama, M. Tsuzuki, H. Satoh and Y. Nakamura (2009) *Chlorella* starch branching enzyme II (BEII) can complement the function of BEIIb in rice endosperm. Plant Cell Physiol. 50: 1062–1074.

Shu, X., J. Sun and D. Wu (2014) Effects of grain development on formation of resistant starch in rice. Food Chem. 164: 89–97.

Stinard, P.S., D.S. Robertson and P.S. Schnable (1993) Genetic isola-tion, cloning, and analysis of a *mutator*-induced, dominant anti-morph of the maize *amylose extender1* locus. Plant Cell 5: 1555–1566.

Sun, C., P. Sathish, S. Ahlandsberg, A. Deiber and C. Jansson (1997) Identification of four starch-branching enzymes in barley endosperm: partial purification of forms I, IIa and IIb. New Phytol. 137: 215–222.

Tanaka, N., N. Fujita, A. Nishi, H. Satoh, Y. Hosaka, M. Ugaki, S. Kawasaki and Y. Nakamura (2004) The structure of starch can be manipulated by changing the expression levels of starch branching enzyme IIb in rice endosperm. Plant Biotechnol. J. 2: 507–516.

Topping, D.L. and P.M. Clifton (2001) Short chain fatty acids and human colonic function-roles of resistant starch and non-starch polysaccharides. Physiol. Rev. 81: 1031–1064.

Wattebled, F., Y. Dong, S. Dumez, D. Delvalle, V. Planchot, P. Berbezy, D. Vyas, P. Colonna, M. Chatterjee, S. Ball *et al.* (2005) Mutants of Arabidopsis lacking a chloroplastic isoamylase accumulate phytoglycogen and an abnormal form of amylopectin. Plant Physiol. 138: 184–195.

Wei, C., F. Qin, W. Zhou, H. Yu, B. Xu, C. Chen, L. Zhu, Y. Wang, M. Gu and Q. Liu (2010) Granule structure and distribution of allomorphs in C-type high-amylose rice starch granule modified by antisense RNA inhibition of starch branching enzyme. J. Agric. Food Chem. 58: 11946–11954.

Wolever, T.M. (2003) Carbohydrate and the regulation of blood glucose and metabolism. Nutr. Rev. 61: S40–48.

Wong, J.M., R. de Souza, C.W. Kendall, A. Emam and D.J. Jenkins (2006) Colonic health: fermentation and short chain fatty acids. J. Clin. Gastroenterol. 40: 235–243.

Xia, H., M. Yandeau-Nelson, D.B. Thompson and M.J. Guiltinan (2011) Deficiency of maize starch-branching enzyme I results in altered starch fine structure, decreased digestibility and reduced coleoptile growth during germination. BMC Plant Biol. 11: 95.

Yang, C.Z., X.L. Shu, L.L. Zhang, X.Y. Wang, H.J. Zhao, C.X. Ma and D.X. Wu (2006) Starch properties of mutant rice high in resistant starch. J. Agric. Food Chem. 54: 523–528.

Yang, L., J. Ding, C. Zhang, J. Jia, H. Weng, W. Liu and D. Zhang (2005) Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. Plant Cell Rep. 23: 759–763.

Yang, R., Q. Tang, H. Wang, X. Zhang, G. Pan, H. Wang and J. Tu (2011) Analyses of two rice (*Oryza sativa*) cyclin-dependent kinase inhibitors and effects of transgenic expression of *OsiICK6* on plant growth and development. Ann. Bot. 107: 1087–1101.

Yang, R.F., C.L. Sun, J.J. Bai, Z.X. Luo, B. Shi, J.M. Zhang, W.G. Yan and Z.Z. Piao (2012) A putative gene *sbe3-rs* for resistant starch mutated from *SBE3* for starch branching enzyme in rice (*Oryza sativa* L.). PLoS ONE 7: e43026.

Yano, M., K. Okuno, J. Kawakami, H. Satoh and T. Omura (1985) High amylose mutants of rice, *Oryza sativa* L. Theor. Appl. Genet. 69: 253–257.

Zhao, Y., N. Li, B. Li, Z. Li, G. Xie and J. Zhang (2015) Reduced expression of starch branching enzyme IIa and IIb in maize endosperm by RNAi constructs greatly increases the amylose content in kernel with nearly normal morphology. Planta 241: 449–461.

Zhu, L., M. Gu, X. Meng, S.C. Cheung, H. Yu, J. Huang, Y. Sun, Y. Shi and Q. Liu (2012) High-amylose rice improves indices of animal health in normal and diabetic rats. Plant Biotechnol. J. 10: 353–362.