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Biomolecular Analyses of Starch and Starch Granule Proteins in the High-Amylose Rice Mutant Goami 2

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Supporting Information

ABSTRACT: Elevated proportions of amylose in cereals are commonly associated with either the loss of starch branching or starch synthase activity. Goami 2 is a high-amylose mutant of the *temperate japonica* rice variety Ilpumbyeo. Genotyping revealed that Goami 2 and Ilpumbyeo carry the same alleles for *starch synthase IIa* and *granule-bound starch synthase I* genes. Analyses of granule-bound proteins revealed that SSI and SSIIa accumulate inside the mature starch granules of Goami 2, which is similar to the *amylose extender* mutant IR36ae. However, unlike the *amylose extender* mutants, SBEIIb was still detectable inside the starch granules of Goami 2. Detection of SBEIIb after protein fractionation revealed that most of the SBEIIb in Goami 2 accumulates inside the starch granules, whereas most of it accumulates at the granule surface in Ilpumbyeo. Exhaustive mass spectrometric characterisations of granule-bound proteins failed to detect any peptide sequence mutation or major post-translational modifications in Goami 2. Moreover, the signal peptide was found to be cleaved normally from the precursor protein, and there is no apparent N-linked glycosylation. Finally, no difference was found in the *SBEIIb* structural gene sequence of Goami 2 compared with Ilpumbyeo. In contrast, a G-to-A mutation was detected in the *SBEIIb* gene of IR36ae located at the splice site between exon and intron 11, which could potentially introduce a premature stop codon and produce a truncated form of SBEIIb. It is suggested that the mutation responsible for producing high amylose in Goami 2 is not due to a defect in *SBEIIb* gene as was observed in IR36ae, even though it produces a phenotype analogous to the *amylose extender* mutation. Understanding the molecular genetic basis of this mutation will be important in identifying novel targets for increasing amylose and resistant starch contents in rice and other cereals.

KEYWORDS: *amylopectin, ion trap tandem MS, Goamy 2, mass spectrometry, matrix-assisted laser desorption/ionization MS, resistant starch, single-nucleotide polymorphism, Suweon 464*

INTRODUCTION

Goami 2 (also known as Goamy 2 and Suweon 464) is a high-amylose mutant resulting from *N*-methyl-*N*-nitrosourea (MNU) treatment of Ilpumbyeo, a high-quality temperate *japonica* rice variety.[1,2] The functional differences separating Goami 2 from Ilpumbyeo include a 2-fold increase in apparent amylose content (AAC) and significantly higher pasting and gelatinization temperatures (GT) of the starch.[3−5] Furthermore, Goami 2 has smaller starch granules,[1,5] B-type starch crystallinity,[3,4] and a lower proportion of short-chain amylopectin.[3] Goami 2 is also reported to have elevated levels of total, soluble, and insoluble dietary fiber (DF), which were found to contribute to weight loss and the reduction of triacylglycerol concentrations in obese subjects.[6] The elevated level of DF in Goami 2 has been attributed to fibrillar cellulose or hemicellulose microfilaments observed in micrographs of raw[1] and cooked[2] rice grains.

The mutation or mutations responsible for the properties of Goami 2 have not been identified at either the genetic or molecular level. Comparison with other mutants in cereals that influence amylose content indicates three possible candidate genes: *granule-bound starch synthase I* (*GBSSI* or *Wx*), *starch synthase IIa* (*SSIIa* or *alk*), and *starch branching enzyme IIb*

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(*SBEIIb* or *ae*). GBSSI is the major starch synthase inside the granules, and this enzyme is essential for amylose biosynthesis.[7−9] A loss of function mutation in the gene responsible for GBSSI leads to glutinous rice grains containing starch exclusively composed of amylopectin.[10] A second candidate, SSIIa, is a major starch synthase in the plastid stroma[7,8] containing natural sequence variations that have been associated with differences in GT and amylopectin structure.[11−15] Loss of function of *SSIIa* leads to high amylose in barley[16] and wheat.[17] Finally, SBEIIb is a major starch branching enzyme in the rice endosperm,[8] and a loss of function mutation in this leads to the *amylose-extender* (*ae*) phenotype in rice[18,19] and in maize.[20] The structural and functional differences observed between Goami 2 and Ilpumbyeo[3,4,21,22] resemble those found between other *ae* and wild-type pairs of rice and maize[18,23−26] and do not parallel changes in starch structure resulting from either *Waxy* or *SSIIa* mutations in rice or other cereals.[10,16,27,28]

In rice, *SBEIIb* mutants have been generated in *temperate japonica*[29−31] and *indica* backgrounds.[23,32] The *amylose extender* mutation in *temperate japonica* background was generated by chemical mutagenesis of Kinmaze,[29] and this mutation was backcrossed into IR36 to introduce the mutation in an *indica* background.[23] Nishi et al.[33] biochemically characterized the *amylose extender* mutation in a *japonica* rice background (EM10), but up to now, the actual molecular genetic basis of its mutation has not yet been fully elucidated. It is clear, however, that when the *ae* mutation is present in the *temperate japonica* background, it has a large effect on both AAC (∼2-fold increase) and GT (10−13 °C increase).[18,25] By contrast, only small increases in both parameters were observed in the *indica* background of IR36, compared with the mutant IR36ae (7% and 1.5 °C, respectively), because AAC and GT are both at the upper ends of those found in the wild-type rice.[23] Varieties of maize with a mutation in *SBEIIb* have higher AAC[34,35] and more resistant starch (RS) than their wild types.[36] RS contributes to the measured DF.[37] Therefore, a mutation in *SBEIIb* is consistent with all of the phenotypic differences observed between Goami 2 and Ilpumbyeo.

In this study, we characterized the starch and starch granule proteins as well as the putative structural gene (*SBEIIb*) of Goami 2 and Ilpumbyeo and compared it with IR36ae and IR36 to determine whether a mutation exists in *SBEIIb* or the other, less likely, candidates, *Wx* and *SSIIa*. We demonstrate here that the mutation in Goami 2 largely phenocopies the *amylose extender* mutation with respect to starch properties. We have located the putative mutation in the *SBEIIb* gene of IR36ae, but no analogous mutation was found in the *SBEIIb* of Goami 2, raising the possibility that causal mutation may be at a different locus.

MATERIALS AND METHODS

Plant Materials and Chemicals. Polished grains of Ilpumbyeo and Goami 2 were a kind gift from the Rural Development Administration (Republic of Korea). IR36 and IR36ae were obtained from Yanco Agricultural Institute (NSW, Australia) and grown at CSIRO Plant Industry (ACT, Canberra, Australia). The ami-BEIIb rice line was recently developed by CSIRO Plant Industry using artificial microRNA-mediated SBEIIb down-regulation in Nipponbare background.[19] Grains were ground to flour to pass through a 0.5 mm sieve.

Reagent grade chemicals, molecular biology grade reagents, and reverse osmosis water filtered through a 0.22 μm Milli-Q filter (Millipore, Billerica, MA, USA) were used throughout the study.

Measurement of Resistant Starch. Samples of both varieties were cooked by the absorption method.[19] A subsample was allowed to retrograde by placing it at 4 °C overnight. Resistant starch was measured in freshly cooked (RS2) and retrograded (RS3) samples after rewarming to room temperature, using the Resistant Starch Assay Kit (Megazyme, Ireland) according to AACC Approved Method 32-40.[38]

Structure of Debranched Starch. The chain-length distributions of debranched starch in the two varieties were determined using fluorophore-assisted capillary electrophoresis (FACE) and size exclusion chromatography (SEC). For both techniques, flour was gelatinized and debranched as previously described.[39] Debranched flour samples were labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS), and FACE was conducted following a previously reported methodology.[40,41] Sample preparation, debranching, labeling, and FACE were performed in triplicate.

Debranched chains of starch were separated using a Waters SEC system (Alliance 2695, Waters, Milford, MA, USA), fitted with an Ultrahydrogel 250 column (Waters) or Proteema 100 (PSS Polymer Standards Service, GmbH, Germany) using ammonium acetate (0.05 M with 0.02% sodium azide, pH 4.75) as eluent. The column was calibrated for molecular weight using pullulan standards (P800, P400, P200, P100, P50, P20, P10, P5) (Shodex, Kawasaki, Japan) injected individually, the Mark−Houwink−Sakaruda equation (K = 0.00126 mL g^{-1} and α = 0.733 for pullulan and K = 0.0544 mL g^{-1} and α = 0.486 for linear starch),[42] and universal calibration.[39,42] Sample preparation and SEC were performed in triplicate.

Genotyping for *SSIIa* and *Wx* Alleles. DNA was extracted from the polished grains using a modified SDS-mini-prep method.[43] Allele-specific primers were used to genotype two single-nucleotide polymorphisms (SNPs) and a dinucleotide polymorphism (a functional nucleotide polymorphism, FNP) in exon 8 of the *SSIIa* gene as described previously.[15]

The SNP at the splices site of intron 1 of the *Wx* gene was identified using previously described methods.[44,45] The products were resolved in 2% agarose gel, stained with SybrSafe nucleic acid stain (Invitrogen, Carlsbad, CA, USA), and visualized using a nonultraviolet transilluminator (Dark Reader DR195M, Clare Chemicals, Dolores, CO, USA).

Proteomic Analyses. Soluble, granule-associated (those that had dried onto the granule surface during desiccation, including those inside the starch granules), and granule-bound proteins (those inside the starch granules that resisted Proteinase K digestion and thorough washing) were sequentially extracted from duplicate flour samples of Goami 2 and Ilpumbyeo according to previously published protocols[46,47] with slight modifications.

Soluble proteins were obtained by suspending flour (0.3 g) in 1 mL of extraction buffer (50 mM Tris, pH 8, containing 10 mM EDTA and 5 mM dithiothreitol), shaken continuously (1 min on a vortex mixer, 15 min on a rotary shaker), and then centrifuged (18000*g*, 5 min). The supernatant, containing the soluble proteins, was transferred to a fresh microfuge tube and stored on ice. The pellet, containing the starch granules, and granule-associated proteins was washed five times with extraction buffer to remove residual soluble proteins. At the end of each wash, the mixture was centrifuged (18000*g*, 5 min) and the supernatant discarded. The pellet was divided into two subsamples of about 100 mg each. The first was suspended in a 1 mL solution of extraction buffer and 50 μg mL^{-1} Proteinase K (Sigma-Aldrich, St. Louis, MO, USA). The suspension was incubated at 37 °C in a water bath (30 min) with regular agitation on a vortex mixer to digest and dislodge granule-associated proteins. The suspension was then centrifuged (18000*g*, 5 min) and the resulting supernatant subsequently discarded. The pellet was washed five times with extraction buffer. Each washing step was followed by a centrifugation step (18000*g*, 5 min), after which the supernatant was discarded. Finally, the pellet was washed with 1 mL of absolute ethanol. The resulting pellet contained starch granules with granule-bound proteins. The second subsample was treated in a similar manner as the first, using extraction buffer without Proteinase K, to obtain granule-associated proteins. The three fractions containing (1) granule-bound

proteins, (2) granule-associated, and (3) the soluble protein extracts were dried using a Speed Vac SC100 (Savant, Sunnyvale, CA, USA).

In each dried fraction (50 mg), proteins were dispersed in 1 mL of gelatinization buffer (50 mM Tris-HCl buffer, pH 8.0, with 10% SDS) and then heated with constant stirring in a boiling water bath (8 min). Granule-bound proteins were also similarly extracted using starch samples (4 mg for *japonica* lines and 2 mg for *indica* lines) and resuspended in 50 μL of gelatinization buffer. Each suspension was then centrifuged (18000*g*, 15 min). The supernatants, containing extracted proteins, were transferred to fresh microfuge tubes; 3 volumes of acetone was added to each supernatant. Proteins were precipitated overnight in acetone at 4 °C then centrifuged (18000*g*, 5 min). Each precipitated protein fraction was resuspended in gelatinization buffer (2 mg μL^{-1} protein, w/v) and heated in a boiling water bath (8 min) prior to loading onto SDS-PAGE gels.

SDS-PAGE. Protein fractions (20 μg per well) were resolved in a Nu-PAGE 4−12% gradient gel (Invitrogen) with 1× MOPS−Tris−SDS buffer in an Xcell SureLock Mini Cell (Invitrogen) operated at 200 V (90 min). Gels were stained with Sypro Ruby (Invitrogen) as per the manufacturer's instructions and visualized with a UV transilluminator (Uvitec, UK). Novex Sharp and BenchMark protein ladders (Invitrogen) were used to estimate the molecular weight of protein bands.

Western Blots. Proteins resolved by SDS-PAGE were electroblotted onto a nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen). The intensity of the electrotransferred BenchMark or Novex Sharp prestained protein ladders (Invitrogen) and Ponceau staining were used to assess the success of electroblotting and the normalization of protein concentration in each well. The Western blot was probed with primary polyclonal antibodies raised against SBEI, SBEIIa, SBEIIb, SSI, SSIIa, SSIIIa, and GBSSI. Proteins were labeled with a goat anti-rabbit immunoglobulin−horseradish peroxidase conjugate (Bio-Rad, Hercules, CA, USA) and visualized using an ECL Western Blotting Detection System (Amersham Pharmacia Biotech, Uppsala, Sweden).

All antibodies used for Western blot in this study are listed in Supporting Table 1 of the Supporting Information, which also describes their dilution, specificity, and source. The production of antibodies raised against wheat SBEIIb,[46] SSI,[48] and GBSSI[47] was described elsewhere. The specificity of these antibodies in detecting their corresponding enzyme isoforms in rice is demonstrated in this paper. Anti-rice SBEIIa was developed using two antigenic peptides: IPAVAEASIKVVAED (peptide 1) or AGAPGKVLVPG (peptide 2). Cysteine and glycine residues were added to these peptides to enable conjugation to either keyhole limpet protein or ovalbumin. These conjugates were used to raise SBEIIa antisera in rabbits. The SSIIa polyclonal antibodies were produced using two similarly conjugated peptides: CGAQDVGIRKYYKA (peptide 1) and CGQDVQLVLLGS (peptide 2). The antiserum raised in rabbits against peptide 2 (AGAPGKVLVPG) conjugated to ovalbumin was found to be the most satisfactory in detecting rice SBEIIa, and the antisera produced using peptide 1−ovalbumin conjugate (CGAQDVGIRKYYKA) proved to be the most specific to rice SSIIa. These two antisera were used for the experiments described in this paper. Finally, affinity-purified rabbit immunoglobulins raised against rice SBEI and barley SSIIIa were produced by Life Research (Burwood East, Victoria, Australia). The specificity of these antisera was assayed by ELISA. The barley SSIIIa polyclonal antibodies effectively detected SSIIIa in rice.

Mass Spectrometry. The protein bands that immunoreacted with the SBEIIb antibody were excised from the gel for mass spectrometric identification. Samples were reduced (25 mM dithiothreitol in 25 mM ammonium bicarbonate), alkylated (55 mM iodoacetamide in 25 mM ammonium bicarbonate), and digested in-gel with trypsin or chymotrypsin for 16 h at 37 °C. The resulting peptides were desalted and concentrated by C_{18} Zip-tip (Millipore) prior to spotting 1:1 with α-hydroxycinnamic acid (CHCA) matrix. In-solution tryptic digestion of total granule-bound protein extracts was also performed as described above to detect all starch enzymes present in the starch granules.

The tryptic peptides were separated by capillary liquid chromatography and identified using an ion trap tandem mass spectrometer (MS) as previously described.[49] Tryptic peptides were analyzed in two ways. First, ions were selected for fragmentation according to the instrument's data-dependent default settings for peptides. Second, ions were selected according to a list of predicted mass-to-charge (m/z) ratios from the predicted masses of peptides with single, double, or triple protonation from particular proteins, including the signal region.

To increase the sequence coverage, tryptic and chymotryptic peptide solutions were lyophilized and reconstituted in 0.1% trifluoroacetic acid and analyzed by liquid chromatography−matrix-assisted laser desorption/ionization (LC-MALDI) on an UltrafleXtreme tandem time-of-flight (TOF/TOF) MS (Bruker Daltonik GmbH, Bremen, Germany). The sample was injected onto an Ultimate 3000 capillary high-performance liquid chromatography (HPLC) system, and chromatographic separation was achieved using a linear gradient of 2−42% acetonitrile over 120 min at 2 $\mu L\ min^{-1}$ on a 200 μm × 5 cm PepSwift Monolithic column (Dionex, Sunnyvale, CA, USA). The eluant was mixed 1:1 with CHCA (5 mg mL^{-1} in 20% ethanol, 70% acetone, 10% ammonium citrate (10 mM) in water) and spotted directly onto a polished steel MALDI target with 22 s fractions. Data were acquired in positive ion reflector mode over the mass range 800−3500 Da using WARP-LC software. The laser power was set to 30%, and 1000 shots were averaged for MS acquisition. Tandem MS spectra were acquired automatically with 2000 shots averaged per spectra.

Bioinformatics Analyses. Ion trap tandem MS data were analyzed using Spectrum Mill software (rev. A.03.03.078, Agilent Technologies) as previously described[49] with the Viridiplantae subset of the NCBInr protein database. LC-MALDI data were searched using both Mascot and ProteinPilot v3.0 software (Applied Biosystems). The subcellular localization of the identified protein was predicted using TargetP,[50] and the signal peptide sequence including its most probable cleavage site[51] was identified by ChloroP.[52] In both cases, the Centre for Biological Sequence (CBS) protein sorting analysis and prediction servers were used (http://www.cbs.dtu.dk/services/index.php).

N-Terminal Amino Acid Sequencing. An extract of granule-bound protein from Goami 2 was separated by SDS-PAGE as described above and electroblotted into a polyvinylidene fluoride (PVDF) membrane (Millipore). The sample was subjected to 30 cycles of Edman N-terminal sequencing using an Applied Biosystems 494 Procise Protein Sequencing System.

Detection of Glycosylation. The granule-bound protein extract from Goami 2 was precipitated in acetone overnight at −4 °C. The protein pellet was obtained by centrifugation at 4 °C for 10 min at 16000*g*. The sample was adjusted to 1 mg mL^{-1} using Peptide N-glycosidase F (PNGase F) buffer (50 mM sodium phosphate, pH 7.5, 0.2% SDS, and 100 mM mercaptoethanol) and deglycosylated using 5 U PNGase F (Sigma) for 3 h in a 37 °C water bath. Mobility shifts were detected by SDS-PAGE using using 4−12% Nu-PAGE gel (Invitrogen) and proteins stained using Quick Coomassie Reagent (Amresco). RNase B (Sigma) was used as a glycosylated protein standard.

Sequencing of the *SBEIIb* Gene. Eleven overlapping primer pairs that amplify the whole *SBEIIb* gene, including its 5′- and 3′-untranslated region (UTR), were designed on the basis of Gramene reference sequence LOC_Os02g32660. Each primer pair was designed to amplify a region of around 1500 bp, overlapping with the next region by 500 bp to ensure proper contig analyses (Supporting Information, Supporting Table 2). The polymerase chain reaction (PCR) products were sequenced (Macrogen, Seoul, Korea). Contigs were generated from the ABI electropherograms from the sequencing results using VectorNTI version 11 software (Invitrogen).

Statistical Analysis. Data were analyzed with balanced analysis of variance (ANOVA) in CropStat (IRRI) for Windows (version 6.1.2007.1). Comparison of means was done using least significant difference (LSD) at the 5% level of significance.

RESULTS

Resistant Starch Content. The functional properties of Goami 2 have already been published elsewhere, but the RS content has not yet been reported. Considering that Goami 2 had elevated amounts of dietary fiber, the RS content of this mutant was determined in this study. RS was significantly higher in both freshly cooked (RS2) and retrograded (RS3) rice of Goami 2 compared with the wild type (Table 1).

Table 1. Determination of Resistant Starch Content in Goami 2 and Ilpumbyeo Using the Megazyme RS Assay Kit

variety	resistant starch 2 (%) (freshly cooked)	resistant starch 3 (%) (retrograded)
Ilpumbyeo	0.98	1.21
Goami 2	6.28	11.45

Genotyping for *SSIIa* and *GBSSI*. Because previous studies speculated that Goami 2 has a defect in its SBEIIb gene, this mutant was genotyped for *GBSSI* and *SSIIa* to immediately rule out these less likely gene candidates. Results revealed that Goami 2 and the wild-type Ilpumbyeo both carried the Wx^b allele of the *Waxy* gene (Figure 1A) and both

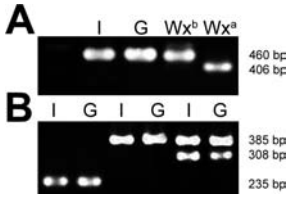

Figure 1. *GBSSI* (*waxy*) and *SSIIa* (*alk*) genotyping in Ilpumbyeo (I) and Goami 2 (G): (A) detection of the G → T polymorphism at intron 1 of the *waxy* gene;[45] (B) PCR products of SNPs 2, 3, and 4 in exon 8 of the *SSIIa* gene.[14].

belonged to haplotype 4 of *SSIIa* (Figure 1B). These alleles code for partially inactive GBSSI and SSIIa, respectively; hence, additional mutations in these starch enzymes could not potentially explain the phenotypic differences observed between Goami 2 and Ilpumbyeo.

Structure of Debranched Starch. The debranched starch structure of Goami 2 and Ilpumbyeo was then determined because it had been previously demonstrated to be useful in providing mechanistic information on starch biosynthesis and to identify mutations in starch enzymes.[40,53,54] Normalized molecular size distribution of debranched starch showed that Ilpumbyeo and Goami 2 have similar concentrations and size distributions of amylose chains of DP ≥1000 (Figure 2A). However, amylopectin size distributions between the two varieties were distinct. On the basis of the demarcations defined for molecular size distribution of debranched starch in rice,[19] Goami 2 had fewer short amylopectin chains (DP 6−36) and more long-chain amylopectin (DP 37−120) and intermediate chains (DP 120−1000) than Ilpumbyeo (Figure 2A). These starch structural properties are expected for rice grains with an *amylose extender* mutation.[19,33]

The differences in the debranched starch molecular size distribution between Ilpumbyeo and Goami 2 were similar between Nipponbare and ami-BEIIb, a transgenic line with down-regulated SBEIIb.[19] These differences were also observed between an *indica* variety IR36 and its SBEIIb mutant, IR36ae, except that IR36ae also had more amylose chains than IR36

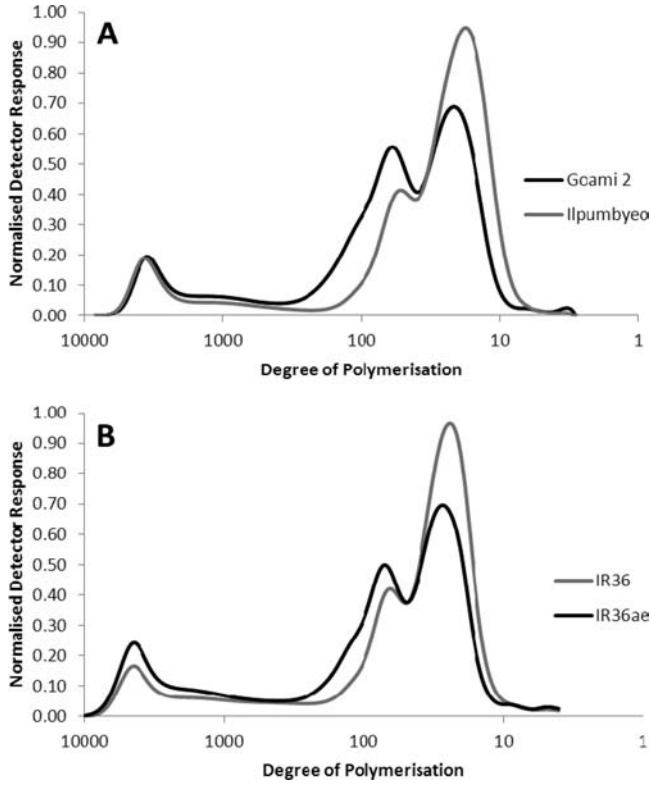

Figure 2. Comparison of normalized molecular size distributions of debranched starch obtained by size exclusion chromatography: (A) Goami 2 and Ilpumbyeo; (B) IR36ae and IR36.

(Figure 2B), probably because they are *indica* lines and, hence, have more active GBSSI. These structural similarities and differences were verified using another size exclusion chromatography column (PSS Proteema 100), which retains chains DP 660 and above in the void volume and provides greater chromatographic resolution for the chains in the amylopectin region (Supporting Information, Supporting Figure 1).

The debranched amylopectin chain length distribution (CLD) profile of Goami and Ilpumbyeo was first reported by Kang et al.,[3] whereas the corresponding profile for *amylose extender* mutants was also reported elsewhere.[18,19] In these previous papers, the mutants have reductions in DP 6−12 chains and elevations in longer chains compared to their wild-type parents. In the present paper, the CLD of Goami was compared to those of the two SBEIIb mutants (ami-BEIIb and IR36ae) to determine whether they all share similar chain lengths despite differences in parental backgrounds. Results revealed that the two SBEIIb mutants shared similar CLDs with Goami 2 (Figure 3).

Western Blot Analyses. Interestingly, although the starch structure and functional properties of Goami 2 grains are homologous to those of the SBEIIb mutants, the approximately 85 kDa SBEIIb polypeptide was still detected by Western blot in Goami 2 but not in the two *SBEIIb* mutants (ami-BEIIb and IR36ae) from total granule-associated proteins extracted from mature starch granules (Figure 4). The amount of SBEIIb in Goami 2 was comparable to that of the three wild types (Ilpumbyeo, IR36, and Nipponbare) (Figure 4).

Because total granule-associated proteins contain both surface-associated and granule-bound polypeptides, protein fractionation was conducted to determine the exact location

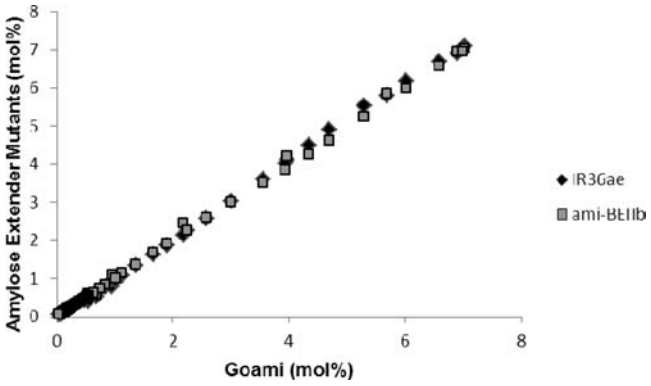

Figure 3. Scatter plot of FACE data comparing the debranched CLD of Goami and the SBEIIb mutants IR36ae and ami-BEIIb from DP 6 to 80.

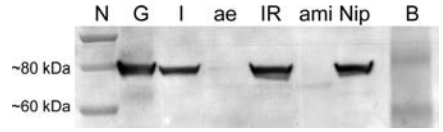

Figure 4. Western blot detection of SBEIIb in the total granule-associated protein fraction of selected mutants and their corresponding parents. SBEIIb was detected in Goami 2 (G), Ilpumbyeo (I), IR36 (IR), and Nipponbare (Nip), but not in the other two SBEIIb mutants IR36ae (ae) and ami-BEIIb (ami). The translucent chemiluminescent film was overlaid on the original nitrocellulose membrane to show the location of Novex Sharp (N) and BenchMark (B) prestained protein ladders.

of SBEIIb in the mature starch granules of Goami 2 and Ilpumbyeo. This revealed that most of the SBEIIb in Goami 2 was trapped inside the starch granules (granule-bound), whereas most of it was present on the surface of the starch granules (surface-associated) in Ilpumbyeo (Figure 5). No SBEIIb was detectable in the soluble fractions of either variety because proteins were extracted from flour samples of mature seeds.

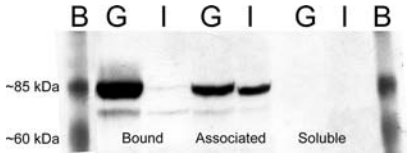

Figure 5. Immunoblot detection of SBEIIb in granule-bound (Bound), granule-associated (Associated), and soluble proteins (Soluble) of Ilpumbyeo (I) and Goami 2 (G). A BenchMark prestained protein ladder (Invitrogen) was used as molecular weight standard (B).

Because elevated amounts of SBEIIb appeared to be trapped inside the mature starch granules of Goami 2, detailed SDS-PAGE and Western blot analyses of granule-bound proteins were conducted to determine what other starch enzymes are present inside the starch granules of this mutant. Interestingly, the intensity of the band corresponding to SBEIIb detected inside the starch granules of Goami appeared similar to that of GBSSI, and these two bands were more intensely stained than the GBSSI detected inside the starch granules of Ilpumbyeo (Figure 6A). In contrast, the amount of putative SBEIIb in Ilpumbyeo was very faint (Figure 6A). Additionally, SSI extracted from inside starch granules stained more intensely

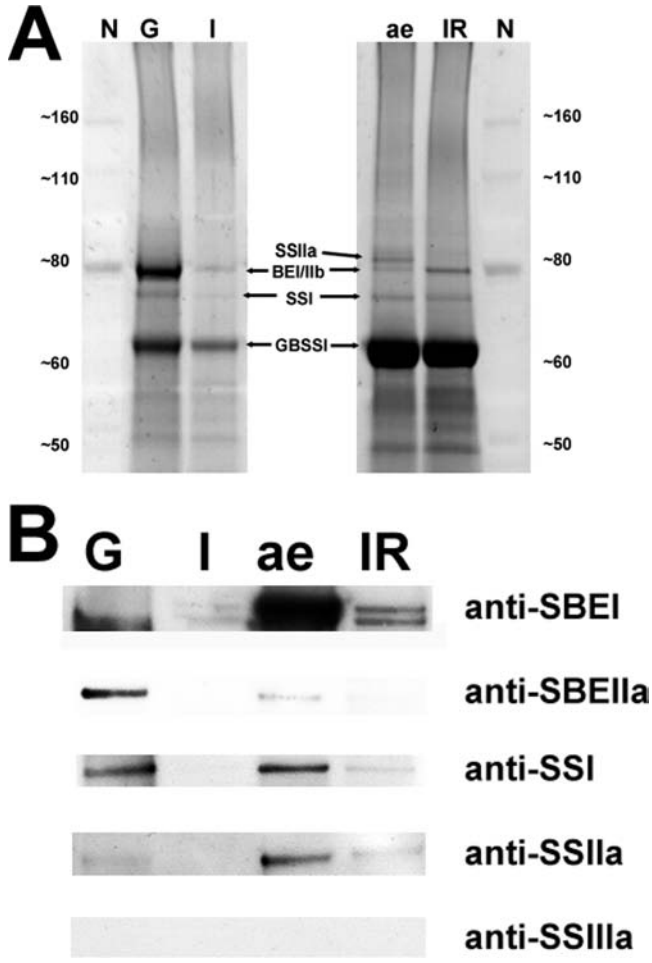

Figure 6. SDS-PAGE (A) and Western blot detection of other major starch enzymes (B) in the granule-bound proteins of Goami 2 (G) and Ilpumbyeo (I) compared with IR36ae (ae) and IR36 (IR). A Novex prestained protein ladder (Invitrogen) was used as molecular weight standard (N).

in extracts from Goami than from Ilpumbeyo (Figure 6A). In comparison, the amounts of GBSSI and SSI in both IR36 and IR36ae appeared to be similar (Figure 6A). Furthermore, the band corresponding to SSIIa was more intensely stained in IR36ae than in Goami 2 (Figure 6A). These observations were verified by Western blots (Figure 6B), which also showed that compared to their respective parents, more SBEI and SBEIIa accumulated inside the starch granules of IR36ae and Goami 2. Western blot analyses also clarified that the faint band detectable at around 85 kDa in IR36ae (Figure 6A) could be due to the accumulation of SBEI inside the starch granules of this mutant (Figure 6B).

Proteomic Analyses. Mass spectrometric analyses of tryptic digests of granule-bound proteins were conducted to verify the results obtained by Western blots, to identify other proteins and peptides not detected by Western blot, to identify possible amino acid differences in the detected peptide fragments, and to detect any differences in posttranslational modifications. SBEI, SBEIIb, and GBSSI peptide fragments were identified in Goami 2 by in-solution and in-gel tryptic and chymotryptic digestion followed by LC-MALDI MS, resulting in >20% sequence identification for each (Supporting Information, Supporting Table 3 and Supporting Figure 2). Three peptide fragments of SSIIa and one fragment of SSI were

1 MAAPASAVPG SAAGLRAGAV RFPVPAGARS WRAAAELPTS RSLLSGRRFP
51 GAVRVGGSGG RVAVR***AAGAS GEVMI***PEGES DGMPVSAGSD DLQLPALDDE
101 LSTEVGAEVE IESSGASDVE GVK**RVVEELA** **AEQKPR**VVPP TGDGQKIFQM
151 DSMLNGYKYH LEYRYSLYRR **LRSDIDQYEG** **GLETFSR**GYE K**FGFNHSAEG**
201 **VTYREWAPGA** **HSAALVGDFN** **NWNPNADR**MS KNEFGVWEIF **LPNNADGSSP**
251 **IPHGSR**VKVR **METPSGIKDS** **IPAWIK**YSVQ AAGEIPYNGI YYD**PPEEEKY**
301 **IFK**HPQPKRP KSLR**IYETHV** **GMSSTEPKIN** **TYANFRDEVL** **PR**IKKLGYNA
351 VQIMAIQEHA YYGSFGYHVT NFFAPSSRFG TPEDLKSLID KAHELGLVVL
401 MDVVHSHASN NTLDGLNGFD GTDTHYFHSG SRGHHWMWDS R**LFNYGNWEV**
451 **LR**FLLSNAR**W** **WLEEYKFDGF** **R**FDGVTSMMY THHGLQVAFT GNYSEYFGFA
501 TDADAVVYLM LVNDLIHGLY PEAITIGEDV SGMPTFALPV QDGGVGFDYR
551 **LHMAVPDKWI** **ELLK**QSDESW KMGDIVHTLT NRRWSEKCVT YAESHDQALV
601 GDKTIAFWLM DKDMYDFMAL DRPATPSIDR GIALHKMIRL ITMGLGGEGY
651 LNF**MGNEFGH** **PEWIDFPR**AP QVLPNGKFIP GNNNSYDKCR R**RFDLGDADY**
701 **LR**YRGMLEFD RAMQSLEEK**Y** **GFMTSDHQYI** **SR**KHEEDKMI IFEKGDLVFV
751 FNFHWSNSYF DYRVGCLKPG K**YKVVLDSDA** **GLFGGFGR**IH HTAEHFTADC
801 SHDNRPYSFS VYSPSRTCVV YAPAE

Figure 7. Mass spectrometry and Edman sequencing of granule-bound SBEIIb from Goami 2. SBEIIb peptide fragments were detected by ESI (underlined) and LC-MALDI (bold) MS/MS sequencing. The location of the signal peptide as predicted by TargetP (highlighted), the point of possible cleavage as predicted by ChloroP (down arrow), and the site of polyclonal antibody detection (boxed) are shown. The N-terminal sequence of the mature protein as detected by Edman sequencing is also indicated (bold italics).

also detected, with 6.0 and 2.8% sequence coverage, respectively. Additionally, several glutelin peptide fragments (Types A1, A3, B1, and B5) were also identified. However, no SBEIIa peptide fragments were detected from the mass spectrometric analyses. Furthermore, only minor modifications, most likely resulting from sample preparation, such as deamidation and oxidation, were detected in some of the peptides identified.

Detailed mass spectrometric analyses were conducted on the SBEIIb of Goami 2. Ion trap tandem MS of in-gel tryptic peptides from granule-bound SBEIIb resulted in the detection of 11 distinct peptides (>95% confidence). The MS/MS spectra confirmed that the 85 kDa protein was SBEIIb with 21% sequence coverage (Supporting Information, Supporting Table 3). LC-MALDI analyses resulted in the identification of SBEIIb with 43% sequence coverage (24 distinct peptides with >95% confidence, Supporting Information, Supporting Table 3). Combining all mass spectrometric results increased the sequence coverage of SBEIIb to 44% (Figure 7), with no significant post-translational modifications detected.

The possibility of uncleaved signal peptide and glycosylation was also investigated to further detect any mutation in the SBEIIb of Goami. Edman sequencing revealed that the first 15 amino acids in the N-terminal sequence of the gel-excised 85 kDa band were AAGASGEVMIPE(G)E(S), the N-terminal sequence of a properly cleaved SBEIIb (Figure 7). Furthermore, peptide *N*-glycosidase F (PNGase-F), which cleaves glycans linked to peptides through asparagine side chains, was used to test for possible differences arising from glycosylation. However, no difference in mobility was observed between the PNGase F-treated and untreated forms of the 85 kDa protein (Figure 8), discounting the presence of N-linked glycans that may somehow interfere with SBEIIb activity or enhance its translocation into starch granules.

Sequencing of the *SBEIIb* Gene. To determine any gene mutation that might have been missed by proteomic analyses, DNA sequencing of Goami 2 and Ilpumbyeo SBEIIb was conducted. The SBEIIb gene of IR36ae and IR36 was also determined for comparison. Gene sequencing showed that the SBEIIb gene of Goami 2 was identical with that of Ilpumbyeo (Figure 9A). No SNP, insertions, or small deletions unique to Goami 2 were detected within the gene, including the exon−

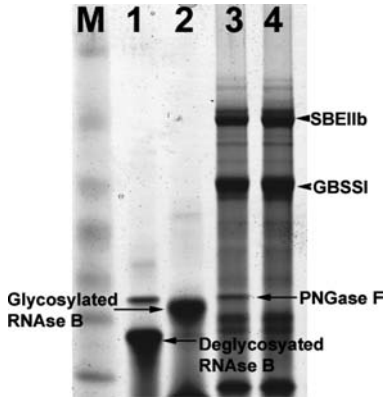

Figure 8. Determination of protein deglycosylation in Goami 2 compared with a glycosylated protein (RNase B) as a control. Lanes show RNAase B with (lane 1) and without (lane 2) treatment with PNGase F (lane 1), and Goami 2 granule-bound proteins with (lane 3) and without (lane 4) treatment with PNGase F (lane 3). Major bands in Goami 2 corresponding to SBEIIb and GBSSI are labeled. A BenchMark prestained protein ladder (Invitrogen) was used as molecular weight standard (M).

intron boundaries. A deletion at position 71 relative to the gene sequence of Nipponbare (Gramene LOC_Os02g32660) was detected in the 5′-UTR, but this was present in both Goami 2 and Ilpumbyeo. In contrast, a G→A SNP mutation was identified in IR36ae that could potentially result in the improper splicing of intron 11 and the introduction of a premature stop codon that could potentially lead to the formation of a truncated SBEIIb (Figure 9B). Prior to the stop codon, this SNP mutation is also predicted to result in a serine to arginine amino acid substitution at position 406. Furthermore, it was observed that the gene sequence of IR36ae *SBEIIb* was more similar to the japonica *SBEIIb* reference sequence (Gramene LOC_Os02g32660) than to that of IR36, which verified the success of genetic introgression of Kinmaze SBEIIb (*japonica*) to an *indica* background (IR36).

DISCUSSION

The similarities of Goami 2's functional and structural properties to those of the rice *amylose extender* phenotype

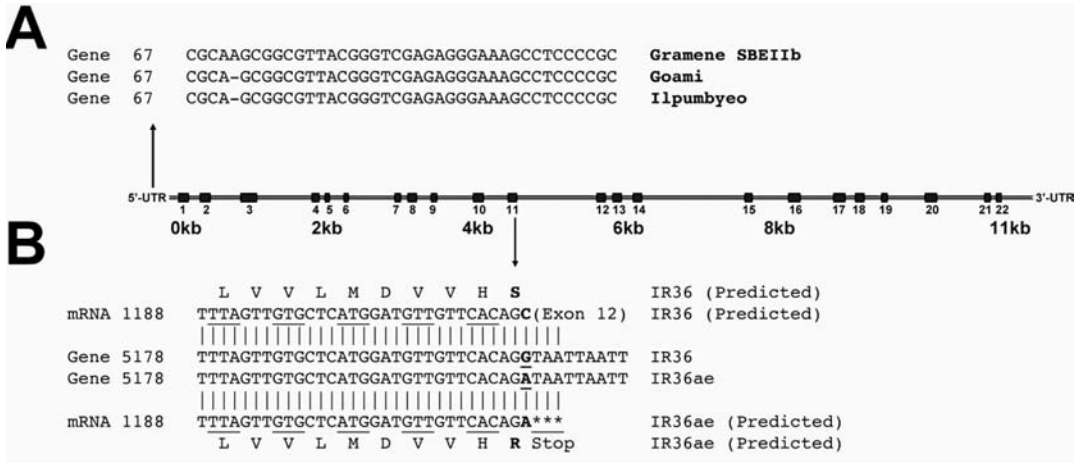

Figure 9. Genomic DNA sequencing of Goami and IR36ae compared with their respective parents: (A) a deletion was observed at position 71 in the 5′-UTR, but this is common to both G and I (boxed inset); (B) aside from an amino acid substitution at amino acid position 406 (serine→arginine), a G→A SNP mutation was observed in IR36ae, which could result in the introduction of a stop codon after exon 11 and potentially result in a truncated protein. Seven overlapping contigs from position 68 to 12295, which spans the 5′- and 3′-UTRs, were aligned, covering 97% of the Gramene SBEIIb reference genomic sequence (Loc_Os02g32660).

suggested that the cause of its mutation is due to a defective SBEIIb.[3,15] However, even though the functional and structural findings of these previous publications were corroborated in this current study (Figures 2 and 3; Supporting Information, Supporting Figure 1), it appears that Goami 2 does not carry a functional mutation in its *SBEIIb* gene as evidenced by the similarity of its structural gene with Ilpumbyeo (Figure 9) and the lack of any detectable posttranslational modifications in its SBEIIb protein sequence (Figures 7 and 8; Supporting Information, Supporting Tables 3 and 4).

In maize,[55,56] pea,[57] and wheat,[47,58,59] SBEs exist in both granule-bound and soluble forms. It was proposed that enzymes accumulate at a constant rate during endosperm development, where about 45% of SBEIIb in maize eventually becomes granule-bound as opposed to 85% of GBSSI.[56] In wheat, the proportion of granule-bound SBEIIb has been estimated to be about a quarter of the total SBEIIb for developing endosperm.[46] There is no similar report in rice, but the amount of SBEIIb protein inside the starch granules of mature wild-type grains was faintly detectable using the conditions described here (Figures 4–6 and 8) and elsewhere.[13] Furthermore, down-regulating the expression of *SBEIIb* in the endosperm of a *japonica* rice line (Nipponbare) using artificial microRNA led to undetectable levels of SBEIIb in the soluble fraction during grain development[19] and even inside mature starch granules (Figure 4, lane 5). The amount of SBEIIb inside the starch granules of some *indica* rice lines is more abundant compared to some selected *japonica* lines,[13] but in the high amylose *indica* mutant IR36ae, where expression of SBEIIb appears to be abolished, the protein was not detected, as expected (Figure 4, lane 3). In contrast, Goami 2 had clearly detectable levels of SBEIIb inside the starch granules (Figures 4–6 and 8), although it is also a high-amylose rice mutant that exhibits phenotypes similar to *indica* and *japonica SBEIIb* mutants. In fact, the level of SBEIIb in the granule-bound fraction of Goami 2 appears to be similar to that of GBSSI (Figure 6) and sharply elevated over what is evident in the parent Ilpumbyeo (Figures 5 and 6). Therefore, it appears that the mutation in Goami 2 results in the trapping and accumulation of SBEIIb inside the starch granules in much greater amounts compared to Ilpumbyeo, which may in turn impede its role in starch biosynthesis if its main function is at the surface of the starch granule. Such aberrant localization may involve other gene(s), regulatory element(s), or post-translational process(es), yet to be identified.

Possible Molecular Genetic Basis of Goami Mutation. Alternative splicing of *SBE2* in kidney bean resulted in two isoforms of SBEII that differ in subcellular localization and affinity for amylopectin, with the smaller BEII (82 kDa) present exclusively in the soluble fraction and the larger form (100 kDa) detectable in both soluble and granule-bound fractions.[60] Moreover, SBEIIb in wheat and maize is known to be phosphorylated at one or more serine residues, and this was found to be crucial in facilitating interaction with other starch synthase and branching enzyme isoforms.[61−65] More recently, a mutant form of SBEIIb in maize (termed *amylose extender 1.2*) was found to be inactive,[66] probably because the mutant protein is truncated as in the case of IR36ae (Figure 9). Interestingly, this inactive form of SBEIIb is found to be hyperphosphorylated, both in the monomeric form and when it is part of a multienzyme starch synthesizing complex, and it is strongly associated with starch granules.[66] In this study, the SBEIIb isoform was found to accumulate inside the starch granule of Goami 2 (Figures 4–6). Because the starch structure of Goami 2 is analogous to the starch structure of *amylose extender* mutants tested in this study (Figures 2 and 3; Supporting Information, Supporting Figure 1) and there is clearly no evidence for a mutation in the Goami 2 SBEIIb protein (Figures 4–8; Supporting Information, Supporting Figure 2 and Supporting Tables 3 and 4) or DNA sequence (Figure 9), it is tempting to speculate that the SBEIIb trapped inside the starch granules of Goami 2 is inactive and probably phosphorylated at multiple sites. It is likely that the Goami 2 SBEIIb is sequestered within the starch granule, away from its normal location where it is active, presumably at the granule surface, as in the case of Ilpumbyeo (Figures 4–6). Experiments to determine the phosphorylation status of both Goami 2 and IR36ae SBEIIb conducted using Pro-Q Diamond (Invitrogen) staining and Phos-Tag (Wako Pure Chemical Industries, Ltd.) gel mobility shift assay were inconclusive (data not shown).

Nutritional Aspect of Goami Mutation. Mutations that lead to disruptions in starch synthesis often are accompanied by an increase in cell wall material and lipids due to the redirection of excess glucose to sinks other than starch.[67] A mutation affecting SBEIIb activity would lead to a decrease in the rate of starch synthesis and could cause glucose to be diverted to other carbon sinks, such as cell wall material. Goami 2 is reported to have a higher concentration of cellulose and hemicellulose than Ilpumbyeo, leading to a higher DF.[1−3,68] The mutation affecting SBEIIb activity could contribute to the increased dietary fiber of Goami 2 both by diverting glucose to cell wall synthesis and by the elevated resistant starch. It is shown here that resistant starch is elevated in Goami 2 (Table 1), which is a common feature of *amylose extender* mutants.[19] The measurement of dietary fiber also captures some of the resistant starch,[37,69−71] suggesting that the nutritional value of Goami 2 might be greater than first expected.

In summary, the structure of starch and the resulting functional properties of Goami 2 are altered in ways consistent with a significant loss of SBEIIb activity, as observed in other amylose-extender rice mutants. However, no mutation was detected in the *SBEIIb* gene or in its protein sequences in Goami 2. Whereas the mutation in Goami 2 has not been precisely identified here, its location in the structural gene for SBEIIb has been ruled out. This study presents information that could lead to the identification of novel targets for mutation to increase amylose and resistant starch contents in rice, which could also be potentially applied to other cereals. Further studies on linkage mapping, as well as molecular genetic and biochemical analyses of this mutation, are required to fully elucidate the basis of the high-amylose trait in Goami 2.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AAC, apparent amylose content; *ae*, amylose extender; ANOVA, analysis of variance; CHCA, α-cyano-4-hydroxycinnamic acid; CLD, chain-length distribution; DF, dietary fiber; DP, degree of polymerization; DSC, differential scanning calorimetry; FACE, fluorophore-assisted capillary electrophoresis; GBSSI, granule-bound starch synthase I; GT, gelatinization temperature; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LSD, least significant difference; MALDI, matrix-assisted laser desorption/ionization; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MS, mass spectrometer or mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; RS, resistant starch; SBEIIb, starch branching enzyme IIb; SSI, starch synthase I; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; SNP, single-nucleotide polymorphism; SSIIa, starch synthase IIa; TOF/TOF, tandem time-of-flight; UTR, untranslated region; *Wx*, *Waxy* gene.

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