Supplemental Methods

Phylogenetic Analysis

Alignments were generated using ClustalW2 (Larkin *et al*., 2007) and then manually optimized. Gap regions were eliminated before phylogenetic analysis. The dendrogram was generated by a neighbor-joining method (*p*-distance model) with 1,000 bootstrap replicates using MEGA software version 6.0 (alignments are given in Supplemental Table S5). Any gaps or missing data were treated using pairwise deletion method. Glycogen synthases (GS) from *Saccharomyces cerevisiae* (Sc GS) were used as an outgroup because eukaryotic glycogen synthases are classified as a different family than starch synthases and prokaryotic glycogen synthases in the CAZy database (Cantarel *et al*., 2007) due to structural and mechanical differences.

The GenBank accession numbers for SS and GS genes are: AtSSI, NP_197818; OsSSI, BAA03739.1; OtSS1, AAS88892; ZmSSI, AAB99957; AtSSII, AAF26156; OsSSIIa, BAD90591; OsSSIIb, AAK81729; OsSSIIc, AAK64284; OtSS2, AAS88880; ZmSSIIa, AAD13341; ZmSSIIb, AAD13342; AtSSIII, AAD30251; OsSSIIIa, AAM49811; OsSSIIIb, AAL40942; OtSS3a, AAS88893; OtSS3b, AAS88894; OtSS3c, AAS88881; ZmSSIII, AAC14014; AtSSIV, CAB78826; OsSSIVa, AAM49812; OsSSIVb, AAM49813; Nos GSalr0031, BAB77555; Syn 6803sll1393, BAL30964; AtSS6, AAK91459; OsSSV, ACC78131; ZmSSV, ACC78132; AtGBSSI, AAN31102; OsGBSSI, CAA44065; OsGBSSII, BAC21549;

OtGBSSI, AAS88890; ZmGBSSI, CAA27574; ZmGBSSII, ABA33603; Nos GSalr1879, BAB73578; CpUWE2, CAF24320; Syn 6803sII 0945, BAL29800; EcGS, AAA23870; ScGS; AAA88715.

Tos17 Mutagenesis and Screening for *ss4b* Lines by PCR

Mutagenesis with *Tos17* and pool sampling were performed as described previously (Hirochika, 2001; Kumar and Hirochika, 2001). To screen for *ss4b*, DNA fragments carrying the *Tos17* transposon were prepared from DNA pools constructed using the three-dimensional sampling method from approximately 40,000 *Tos17*-containing plants. These DNA fragments were subjected to PCR with transposon-specific primers and *OsSSIVb*-specific primers (see Supplemental Figure S2B).

Analysis of Endosperm Starch Crystallinity

X-ray diffraction analysis of endosperm starch crystallinity was performed as described previously (Fujita et al., 2003, 2006, 2007).

Seed Lipid Composition and Levels

Metabolite profiling using gas-chromatography-time-of-flight-mass spectrometry (GC-TOF-MS) was performed as described in Kusano et al. (2007) with minor modifications. Briefly, 100 healthy kernels of wild-type (cv. Nipponbare) and double mutant (*ss3a ss4b*, *#2012*) rice were selected and then the husks were separated

from the kernels. The brown rice grains were polished to obtain bran powder and the polished rice residue. After crushing the polished rice, we prepared three analytical replicates of bran (10 mg fresh weight) and polished rice powder (50 mg fresh weight) for GC-MS and LC-MS analysis, respectively. Bran powder and polished rice powder was extracted with extraction solvent [methanol/chloroform/water (3:1:1, v/v/v)] at a concentration of 10 mg/ml for bran extraction and 100 mg/ml for polished rice extraction. The extraction solvent contained 10 stable isotope reference compounds. After centrifugation, a 50 µl aliquot of the bran supernatant and a 200 µl aliquot of the polished rice supernatant was used for further analysis. We used *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) for trimethylsilylation (Tokyo Chemical Industry, Tokyo, Japan) and the Rxi-5 Sil MS column (30 mm $\times$ 0.25 mm inner diameter fused-silica capillary column with a chemically bound 0.25 µl film) for this analysis. Raw data were exported, normalized, and then concatenated using CCMN and MetMask as described in Redestig et al. (2011). We used R v2.14.2 (64 bit) to conduct CCMN and LIMMA for metabolite profiling analysis.

Protein Extraction from Developing Endosperm for Estimation of GBSSI Protein

Mature rice endosperm was extracted as described by Fujita et al. (2006). Total proteins were prepared by combining the soluble, loosely bound, and tightly bound proteins. The protein extracts were subjected to immunoblot analysis with rice

GBSSI antibody (Fujita et al., 2006). Densitometric analysis of immunoreactive bands was performed using NHI ImageJ 1.44 software to assess GBSSI protein levels.

Preparation of Total Protein Extracts and Immunoblotting of Developing Seeds at Intermediate Stages

Frozen seeds (12 DAF) from wild-type Nipponbare and mutant lines were ground in 300 μL of sample buffer (0.125 M Tris·HCl, pH 6.8, 8 M urea, 4% SDS, 5% 2-mercaptoethanol) per seed with a plastic pestle. Samples were extracted overnight at room temperature (28°C) on a rotating platform and centrifuged at 20,000 × *g* at room temperature for 5 min to remove starch. The supernatants (200 μL) were supplemented with 100 μL of 0.1 M Tris·HCl, pH 6.8, 10% SDS, 12% 2-mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue, and stored at –30°C. Proteins were extracted from seeds at 7 DAF as described by Yun et al. (2011) with the following modifications. Four hulled seeds were weighed immediately after harvest and ground with a mortar and pestle in 50 mM Tris·HCl (pH 6.8), 8 M urea, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20 % (v/v) glycerol, and 0.1% (w/v) bromophenol blue (35 μL/mg). The extracts were vigorously mixed for 30 min at room temperature and centrifuged at 10,000 × *g* for 3 min at room temperature, and the supernatants were stored at –20°C.

Protein extracts (15 μL for 12 DAF; 7 μL for 7 DAF) were separated in 7.5% (w/v) or 4% to 20% (w/v), respectively, gradient gels (Bio-Rad). Proteins were transferred

89 onto PVDF membranes (Millipore or Bio-Rad). The membranes were probed with
90 antibodies against recombinant SSIVb, FtsZ1, FtsZ2-1, MinD, MinE, and ISA3 as
91 described previously (Yun and Kawagoe, 2010; Yun et al., 2011) or an antibody
92 against SSIVa Goat anti-rat antibodies conjugated with horseradish peroxidase
93 (Bio-Rad or KPL) were used for chemiluminescence assays.
94
95 **Generation of a Polyclonal Antibody against SSIVa**
96 The DNA fragment encoding A703–T975 of rice SSIVa was amplified by PCR
97 using the full-length cDNA (AK100976) as template and the primers
98 5′-ATACCATGGCTTTAGAAGTGCGCTCA-3′ and
99 5′-TATCTCGAGTGTTTGAGCCGCTCCCCT-3′. The PCR product was cloned into
100 pET23d (Novagen). The polypeptide was expressed in *E. coli* Rosetta2 (DE3),
101 purified from inclusion bodies by Ni-NTA Sepharose (Qiagen), and injected into a rat
102 for antibody production. The serum was used for western blotting.
103
104 **Cloning and Expression of *OsSSIVb* in *E. coli***
105 The full-length cDNA of *OsSSIVb* was amplified by PCR with the primers
106 5′-CAGCCTCCGCATCCGATTCC-3′ and
107 5′-TGTGGCATCAGCGGCCGCGTCAGAGAAAG-3′. The PCR product was
108 digested with *Nco*I and *Not*I, and cloned into the same sites of *pET30c* to add an
109 N-terminal histidine tag. The construct was confirmed by DNA sequencing. The
110 control plasmid was then prepared from this construct by digestion with *Sal*I and

*Xho*I, and self-ligated to remove the catalytic domain. These plasmids were expressed in *E. coli* BL21 (DE3) containing the pKJE7 chaperone plasmid. Soluble SSIVb proteins were bound to Ni-NTA Sepharose (Qiagen) in a buffer containing 50 mM Tris·HCl (pH 8.0), 0.3 M NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, and 10% glycerol; washed with 50 mM Tris·HCl (pH 8.0), 0.3 M NaCl, 30 mM imidazole, 10 mM 2-mercaptoethanol, and 10% glycerol; and eluted in 50 mM Tris·HCl (pH 8.0), 0.3 M NaCl, 250 mM imidazole, and 10 mM 2-mercaptoethanol. The eluate was concentrated using Amicon Ultra (30-kDa cutoff).

_Os_SSIVb Activity Staining

Approximately 100 ng of purified recombinant protein or 10 μL of rice extract was loaded on a 7.5% (w/v) native PAGE gel with or without 0.05% potato amylopectin (Sigma) as a primer for glucan synthesis. Gels were incubated in reaction buffer containing 0.5 M citrate and 1 mM ADP-glucose overnight at 30°C, and stained with solution containing 1% I_2 and 0.1% KI (Nishi et al., 2001).

Generation of a Polyclonal Antibody against SSIVb

Ni-NTA (Qiagen) purified and C-terminus truncated rice SSIVb was prepared as described above, and then subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining. The SSIVb protein band was excised and electro-eluted (Bio-Rad). The eluted proteins were injected into a rabbit for antibody generation. The serum was used for immuno-electron microscopy analysis.

References for Supplemental Data

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