Critical roles of soluble starch synthase SSIIIa and granule-bound starch synthase Waxy in synthesizing resistant starch in rice

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Changes in human lifestyle and food consumption have resulted in a large increase in the incidence of type-2 diabetes, obesity, and colon disease, especially in Asia. These conditions are a growing threat to human health, but consumption of foods high in resistant starch (RS) can potentially reduce their incidence. Strategies to increase RS in rice are limited by a lack of knowledge of its molecular basis. Through map-based cloning of a RS locus in *indica* rice, we have identified a defective soluble starch synthase gene (*SSIIIa*) responsible for RS production and further showed that RS production is dependent on the high expression of the *Waxy*^{a} (*Wx*^{a}) allele, which is prevalent in *indica* varieties. The resulting RS has modified granule structure; high amylose, lipid, and amylose–lipid complex; and altered physicochemical properties. This discovery provides an opportunity to increase RS content of cooked rice, especially in the *indica* varieties, which predominates in southern Asia.

diabetes | resistant starch biosynthesis | soluble starch synthase | granule-bound starch synthase | amylose–lipid complex

Increases in the incidence of type-2 diabetes are being observed throughout the world. This increase is thought to be due to changes in both diet and lifestyle (1, 2) and is increasingly apparent in Asia. Consumption of foods high in resistant starch (RS) can help to control type-2 diabetes, because its slow digestion and absorption by the small intestine decreases postprandial glucose and insulin responses (3). Foods high in RS also potentially protect against pathogen infection, diarrhea, inflammatory bowel disease, colon cancer, and chronic renal and hepatic diseases. Consumption of RS can increase satiety and reduce calorie intake to help weight management (3). Thus, improvement of the amounts and properties of RS in foods is an important goal.

Rice (*Oryza sativa* L.) is consumed by more than half the world's population (4), and for many, it is the primary source of nutrients and carbohydrates for energy. Consumption of 18–20 g of RS (5, 6) is recommended per day for health benefits, but hot cooked rice typically contains <3% RS (7). Rice varieties or mutants with improved RS have been identified, such as Goami No. 2, Gongmi No. 3, RS111, and Jiangtangdao 1 (7–10).

A high-RS, high-amylose transgenic rice line has been developed by suppressing the expression of starch branching enzymes (SBEs) (11) and a mutation of *SBEIIb* cosegregated with RS content in rice (8). In other cereals, down-regulation of soluble starch synthase (SS) *SSIIa* and of *SBE* results in greater RS in barley (12, 13) and wheat (14–20). Because the molecular basis underlying RS production is largely unknown, discovery of new RS genes is vital both for the elucidation of RS biosynthesis and for the breeding of high-RS varieties. We therefore screened a mutagenized population of the hybrid-rice restorer line R7954 for mutants with high RS in hot cooked rice. This strategy was designed to identify new RS genes of practical value in commercially relevant *indica* rice varieties. Here we report the characterization of one such mutant, revealing that mutations in the starch synthase IIIa (SSIIIa) gene, in combination with a highly expressed Waxy (Wx) gene, lead to a high level of RS.

Results

Mutation of Soluble Starch Synthase Gene *SSIIIa* Results in RS Elevation in a Mutant, *b10*. To find new genes for RS, we screened a population of gamma-radiated hybrid-rice restorer line R7954 and identified a mutant, *b10*, which confers high RS in cooked rice (Fig. 1 *A* and *B*). To clone the *b10* gene, we took a map-based cloning approach (Fig. 1*C*). In the F_2 population, plants with high RS segregated from plants with low and intermediate RS in a 1:3 ratio (13:49; $\chi^2 = 0.54$; $P = 0.46$), suggesting that a single loss-of-function gene is responsible for RS (Fig. 1*B*). The target gene was first located on chromosome 8, and then a larger-scale linkage analysis of 412 plants, segregating for RS, was performed, locating the gene in a 456-kb region between the M6 and M8 markers (Fig. 1*C*). The Gramene Database (www.gramene.org/) predicts 76 protein-coding genes in this region, one encoding a soluble starch synthase (*SSIIIa*; LOC_Os08g09230).

Genomic sequence analysis showed that *SSIIIa* in R7954 encompasses ~10 kb, comprising 16 exons and 5,367 bp encoding a predicted protein of 1,788 amino acid residues. Comparing *SSIIIa* DNA sequence between R7954 and *b10* revealed a G-to-A mutation at the 3′ splice site of intron 5 in *b10* (Fig. 1*D*). This mutation is predicted to result in a novel splice site, leading to a 4-bp deletion in the *SSIIIa* coding sequence and a frame shift

Significance

Resistant starch (RS) has the potential to protect against diabetes and reduce the incidence of diarrhea, inflammatory bowel disease, colon cancer, and chronic renal and hepatic diseases. In this study, we identified two critical starch synthase genes which together regulate RS biosynthesis in rice, and we explored their potential interactions as part of a network of starch biosynthetic enzymes. The findings hold promise for applications in breeding varieties with improvement of RS in hot cooked rice and may also have general implications for understanding RS biosynthesis in other major cereal crops.

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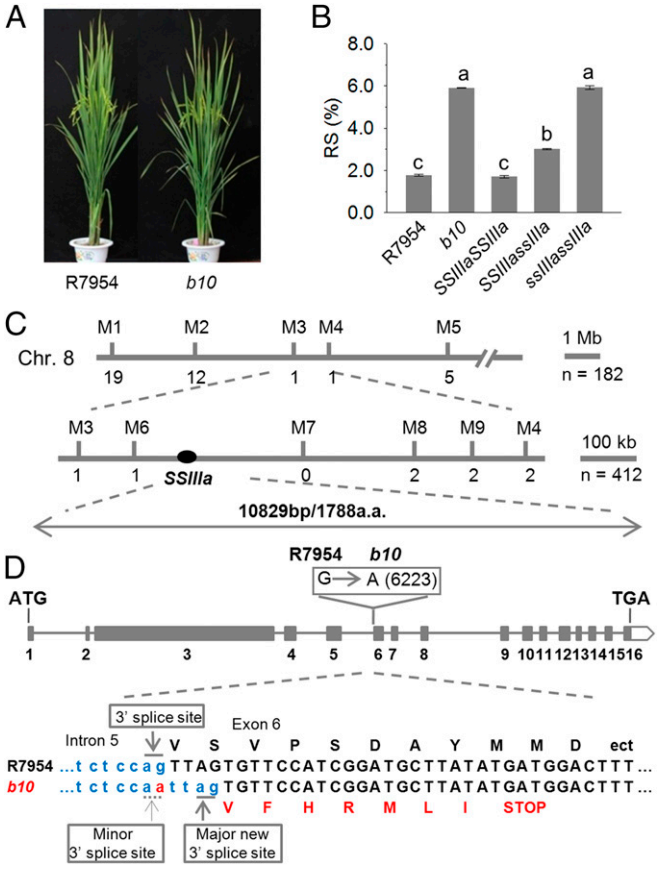

Fig. 1. Characterization of the RS mutant *b10* and positional cloning of the *B10* gene. (*A*) Plant phenotype of the wild type (R7954) and the high-RS mutant (*b10*). (*B*) RS contents of grains from R7954 and *b10* and from plants carrying different *SSIIIa* alleles in an F_2 population from a cross between R7954 and *b10*. Error bars represent ±SEM ($n = 62$). Different letters above bars indicate significant differences at $P < 0.05$, using Tukey's multiple comparison test. (*C*) Mapping of the target gene between the markers M6 and M8 on the short arm of chromosome 8. Numbers below the lines indicate the number of recombinants between the locus and the markers shown. (*D*) *SSIIIa* gene structure and mutation site. Filled boxes indicate exons (numbered 1–16) of *SSIIIa*. Site of the mutation from G to A in *SSIIIa* of *b10* is shown in the open box above exon 6. Nucleotide sequences of the junction between intron 5 and exon 6 in R7954 and *b10* are shown in *Lower*, with deduced amino acid sequences. The mutated nucleotide in *b10* is shown in red, together with the loss of original 3′ splice site and creation of a new 3′ splice site. The mutation generates a recognition site for *Mlu*C I (AATT), which is used to generate a CAPS marker (Fig. S1) to determine genotypes of the plants shown in *B*.

introducing a premature stop codon and a truncated protein of 1,302 amino acid residues. Sequence analysis of cDNA from *b10* confirmed the new splicing site and frame-shift (Fig. 1*D*). The G-to-A mutation introduced a *Mlu*C I restriction enzyme site at the 3′ splice site of intron 5 in *b10*. This site was used to digest a 282-bp fragment obtained by PCR on genomic DNA of a F_2 population from *b10* crossed to R7954 to show cosegregation of the *ssIIIa* gene and RS (Fig. S1). The homozygous mutant *ssIIIassIIIa* plants had 5.8% RS, three times more than that of wild-type R7954 plants (*SSIIIaSSIIIa*), whereas heterozygotes (*SSIIIassIIIa*) had intermediate RS of 3.3%, indicating partial dominance of *SSIIIa* (Fig. 1*B*).

Biosynthesis of RS Is Regulated by *SSIIIa* in Rice. To confirm that the *ssIIIa* gene is responsible for high RS, *b10* was transformed with a 15.6-kb wild-type genomic fragment containing the entire gene (*gSSIIIa*) and with a cDNA driven by a rice *Ubiquitin* promoter (*Ubi:cSSIIIa*). In both cases, the RS content was lowered to wild-type levels (Fig. 2*A*). We further showed that suppression of *SSIIIa* expression in R7954 using RNA interference (RNAi) recapitulated the *b10* phenotype by increasing RS content (Fig. 2*A*). In all cases, changes in *SSIIIa* gene expression were confirmed by quantitative RT-PCR (qRT-PCR) and immunoblotting (Fig. 2 *B* and *C*). A very faint band of SSIIIa detected in immunoblots of *b10* proteins (Fig. 2*C*) suggested that inefficient splicing at the original 3′ splice site occurs with low efficiency (Fig. 1*D*).

For further confirmation, two T-DNA mutants were characterized, one in the Dongjin (DJ) variety and one in Zhonghua 11 (ZH11), both with insertions in the 11th exon of the *SSIIIa* gene (Fig. S2*A*). The mutant in the DJ variety was previously described as the *white-core floury-endosperm* mutant *flo5-1* (21). The two homozygous T-DNA insertion mutants increased RS content from ∼1% to 4.2% and 3.5%, respectively (Fig. S2 *B* and *C*), consistent with a fourfold increase from ∼1.5% to nearly 6% in *b10* (Fig. 2*A*), but the absolute RS contents in both DJ and ZH11 mutants were significantly lower than that in *b10*. Because the DJ and ZH11 varieties are both *japonica* subspecies, the differences in RS content might be attributed to an interaction between the *ssIIIa* allele and genes that differ between *japonica* and *indica* (see below).

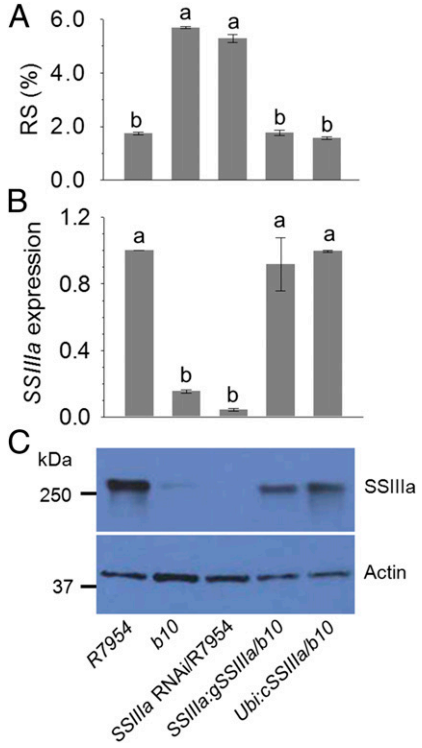

Fig. 2. Confirmation of *SSIIIa* as responsible for the RS phenotype of *b10*. (*A*) RS contents of the wild-type R7954, the mutant *b10*, an *SSIIIa* RNAi line in the R7954 background in which the transgene is driven by the ubiquitin promoter (*SSIIIa* RNAi/R7954), the *b10* mutant complemented with a genomic fragment covering the complete *SSIIIa* wild-type gene (*SSIIIa:gSSIIIa/b10*), and the *b10* mutant complemented with a *SSIIIa* cDNA driven by the ubiquitin promoter (*Ubi:cSSIIIa/b10*). Error bars indicate ±SEM ($n = 3$). (*B*) qRT-PCR of *SSIIIa* expression levels in RNA isolated from developing seeds of the genotypes described in *A*. Reference gene was *Actin*, and results are presented relative to the expression level in R7954. Different letters above bars indicate significant differences at $P < 0.05$, using Tukey's multiple comparison test. (*C*) Immunoblotting of protein isolated from developing grains of the genotypes described in *A* and probed with polyclonal antibodies raised against rice SSIIIa (*Upper*) and actin (*Lower*). Molecular mass markers are shown on the left.

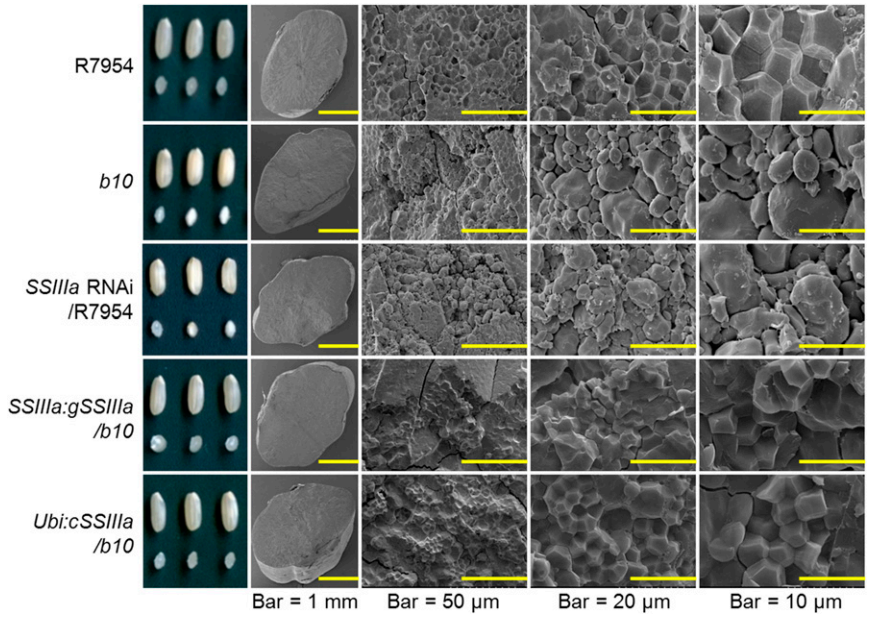

Fig. 3. Morphology of seeds and endosperm from plants with different *SSIIIa* genotypes. Representative samples of intact seeds and transverse sections revealed by light microscopy are shown in *Left*. Scanning electron micrographs of the endosperm in transverse sections are shown with increasing magnification from left to right. Genotypes are the same as shown in Fig. 2.

Grain Qualities and Physicochemical Properties of Starch Are Determined by *SSIIIa*. Examination of polished grains by light microscopy showed that *b10* and *SSIIIa* RNAi transgenic lines have a floury appearance, compared with R7954 and transgenic lines expressing *SSIIIa* (Fig. 3). Scanning electron microscopy (SEM) of fractured surfaces of grains of R7954 revealed similarly sized polygonal starch granules with sharp edges, smooth flat surfaces, and compound starch granules (Fig. 3). In contrast, the granules in *b10* were rounded, variable in size and shape, and with irregular surfaces. Granules in transgenic lines expressing *SSIIIa* appeared similar to those of R7954, whereas those of *SSIIIa* RNAi transgenic lines were similar to those of *b10* (Fig. 3). These observations are consistent with those made for *flo5* mutants (21).

Starch from *b10* and *SSIIIa* RNAi grains showed increased apparent amylose content (AAC), reduced peak viscosity (PV),

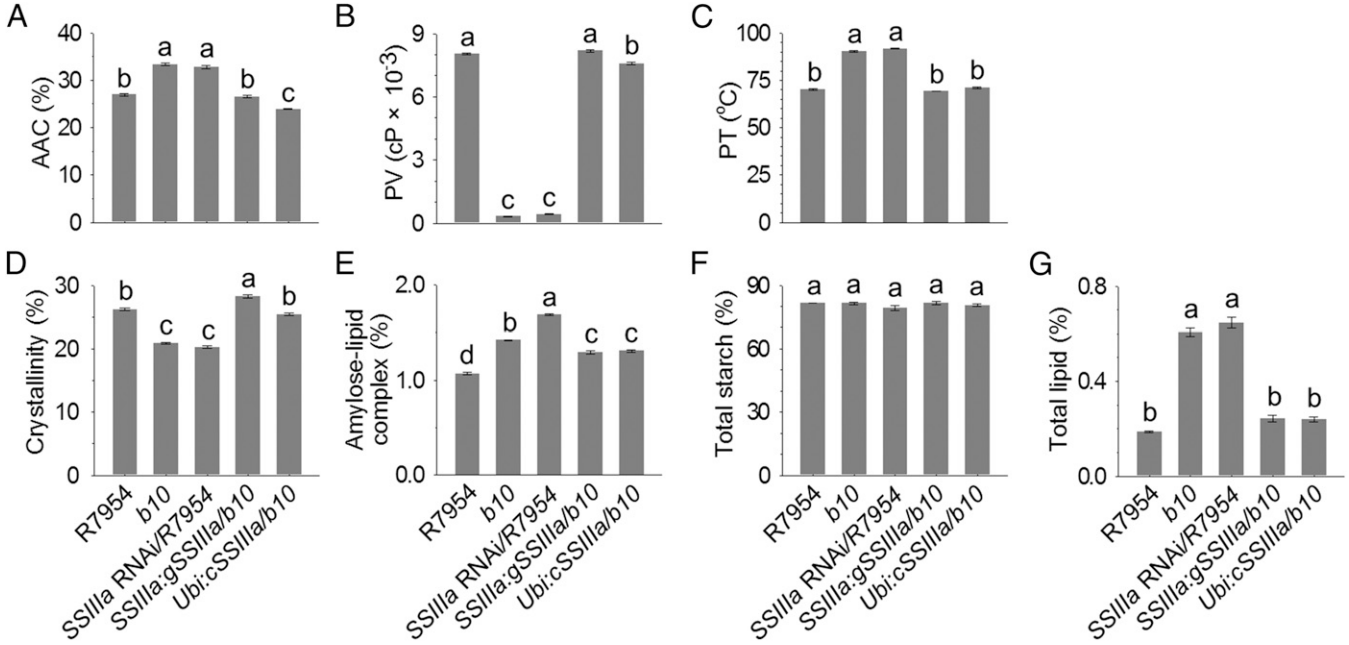

Fig. 4. Physicochemical properties of starch from grains of plants with different *SSIIIa* genotypes. (*A*) AAC expressed as a percentage of dry weight. (*B*) PVs measured in centipoise (cP). (*C*) PTs. (*D*) Crystallinity values determined from X-ray diffraction patterns. (*E*) Content of amylose–lipid complex determined from X-ray diffraction patterns. (*F*) Total starch content of grains. (*G*) Total lipid content. Starch was isolated from mature grains. Genotypes are the same as described in Fig. 2. Error bars show ±SEM. Different letters above bars indicate significant differences at $P < 0.05$ ($n = 3$), using Tukey's multiple-comparison test.

and elevated pasting temperature (PT) (Fig. 4 *A*–*C*), which are also mainly determined by *Wx* (22) and *ALK* (23) genes. These properties were restored to wild type in *b10* lines transformed with *SSIIIa* genes, demonstrating that *SSIIIa* is a key determinant of starch quality (Fig. 4 *A*–*C*). Starch from T-DNA mutants showed similar changes in AAC, PT, and PV (Fig. S3 *A*–*C*), and these findings are consistent with the previous report of increased amylose in starch of *flo5* mutants (21). The increase in PT is consistent with previous report in *SSIIIa* RNAi (24), whereas the reported decrease in gelatinization temperature (21) is apparently inconsistent with the observed increase in PT (Fig. S3*C*); these results could be attributed to the use of different techniques of differential scanning calorimetry and Rapid Visco Analysis, respectively.

Isolated starch was analyzed by X-ray diffraction (Fig. S4), revealing that *b10* and *SSIIIa* RNAi lines had lower crystallinity (Fig. 4*D*) and greater amylose-lipid complex (Fig. 4*E*), as did the T-DNA mutants (Fig. S3 *D* and *E*). This observation is important because amylose–lipid complex constitutes RS type 5 (25). Analysis of chain lengths of amylopectin separated from amylose and then debranched revealed only minor differences between genotypes (Fig. S5), consistent with previous results obtained for *ssIIIa* mutants (21, 26). The amount of starch was not altered in grains of *b10* and *SSIIIa* RNAi transgenic lines (Fig. 4*F*) or was slightly reduced by up to 3% (wt/wt) in T-DNA mutants (Fig. S3*F*), whereas total lipid increased twofold to threefold (Fig. 4*G* and Fig. S3*G*), consistent with the increase in amylose–lipid complex.

Increased RS Content Mediated by *SSIIIa* Requires High Expression Level of the *Waxy* Gene That Encodes Granule-Bound Starch Synthase I. The *Wx* gene encoding granule-bound starch synthase I (GBSSI) has two major alleles, Wx^a and Wx^b, which occur predominantly in *indica* and *japonica* subspecies, respectively (27). The *japonica* Wx^b allele carries a substitution mutation at the 5′ splice site of the first intron, which reduces the amounts of *Wx* mRNA and GBSSI in developing endosperm (28, 29). Genetic interactions between *SSIIIa* and *Wx* genes are known to influence amylose content in *japonica* rice (26). We therefore analyzed the *Wx* genotype of the F_2 populations from a cross between homozygous ZH11 (*SSIIIa*, Wx^b) and *b10* (*ssIIIa,* Wx^a). Combining the homozygous *ssIIIa* mutant with homozygous *indica* Wx^aWx^a alleles resulted in high RS (6.1%), whereas the heterozygous Wx^aWx^b alleles had 5.4% and the homozygous *japonica* Wx^bWx^b alleles resulted in 2.6% RS (Fig. 5*A*). Immunoblotting analysis confirmed the low amount of GBSSI protein in Wx^bWx^b progeny (Fig. 5*B*). The highly significant correlation (0.47; $P < 0.0001$) between RS and *Wx* strongly suggested that RS variation among plants carrying *ssIIIa* arises from the different *Wx* alleles. To confirm that high-level expression of the *Wx* gene is required for RS production, *b10* was transformed with a *Wx* RNAi construct. Two *b10* lines showing strong silencing of *Wx* at RNA and protein levels (Fig. 5 *C* and *D*) showed lower levels of AAC and RS (Fig. 5 *E* and *F*), and also showed higher PV and crystallinity with lower amylose–lipid complex content (Fig. S6). These results confirm the importance of *Wx* in RS production.

Previous studies of an *ssIIIa* mutant in *japonica* reported that the mutation leads to an increase in the expression of the Wx^b gene, an increase in the amount of GBSSI protein, and a 1.3-fold increase in the amount of amylose (26). In a subsequent study, a Wx^a transgene was introduced into a *japonica* background, leading to a high level of GBSSI protein as expected, but the expression level was not further increased in a homozygous *ssIIIa* mutant, potentially because the GBSSI level was already maximal (30). However, the amylose content did increase in a *ssIIIa* background, suggesting an additional posttranslational control of amylose accumulation. Because our genetic analysis revealed an interaction between the *ssIIIa* mutation and the *Wx* allele in RS formation, we investigated whether *ssIIIa* affects *Wx* expression in the *indica* background. The level of *Wx* RNA was measured by qRT-PCR and the amount of GBSSI protein by immunoblotting in *SSIIIa* and *ssIIIa* backgrounds. The *ssIIIa* mutation did not significantly increase the amount of GBSSI RNA or protein (Fig. S7 *A* and *B*), implying that any interactions were likely to occur at the posttranslational level. Furthermore, we analyzed expression of many other genes of starch metabolism in *b10* relative to R7954, but observed only minor or moderate changes in expression (Fig. S7*C*).

Discussion

The high AAC content of starch from plants deficient in *SSIIIa* could result from increases in amounts of both amylose and extralong chains in amylopectin (26, 31). Furthermore, it is

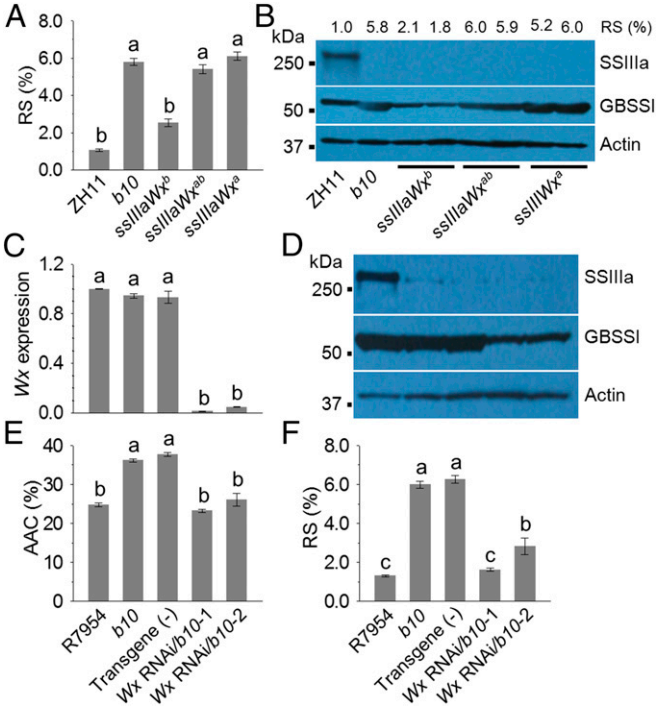

Fig. 5. Effect of different *Wx* alleles on RS production. The segregating F_2 progeny of a cross between *b10 (ssIIIa*, Wx^a*)* and ZH11 (*SSIIIa*, Wx^b) were screened for plants homozygous for *ssIIIa,* and homozygous for either Wx^a or Wx^b alleles, or heterozygous (Wx^{ab}). (*A*) RS contents in grains from plants carrying different *Wx* alleles. Error bars indicate ±SEM ($n = 84$). (*B*) Protein levels in grains from plants were detected by immunoblotting using antibodies recognizing SSIIIa, GBSSI and Actin. The wild-type ZH11 and mutant *b10* were analyzed together with two independent lines (1 and 2) of each *Wx* genotype. Molecular mass (kDa) markers are shown on the left. The RS content of the seeds of these plants was also analyzed and is shown above the immunoblot. (*C*) *Wx* RNA levels in RNA isolated from developing grains, determined by qRT-PCR relative to the *Actin* reference gene, and results are expressed relative to R7954. Error bars indicate ±SEM ($n = 3$). (*D*) Immunoblotting of SSIIIa, GBSSI, and Actin in developing grains from these plants. Molecular mass markers are shown on the left. (*E*) AAC expressed as a percentage of dry weight. (*F*) RS contents of grains from these plants expressed as percent (wt/wt). Starch was isolated from mature grains. Error bars represent ±SEM ($n = 3$). Different letters above bars indicate significant differences at $P < 0.05$, using Tukey's multiple comparison test. For *C*–*F*, mutant *b10* was transformed with a *Wx* RNAi transgene driven by the ubiquitin promoter to reduce *Wx* expression. Two independent lines with low *Wx* RNA level were shown (*Wx* RNAi/*b10*-1 and *Wx* RNAi/*b10*-2), whereas a line with no transgene (–) together with R7954 and *b10* served as controls.

known that high amylose can contribute to RS through the formation of inclusion complexes with lipids (3). The presence of amylose–lipid complex in starch granules restricts their swelling during cooking and thus increases granule resistance of hydrolytic enzymes (25). Consistent with this explanation for increased RS, we observed increased levels of total lipid and amylose–lipid complex in the starch of *b10* (Fig. 4 *G* and *E*). Furthermore, the chain-length distributions in amylopectin exhibited a small increase in the abundance of branches with degrees of polymerization (DP) in the range 10–20 and a small decrease in chains with DP in the range 35–50 (Fig. S5). These changes to amylopectin might also contribute to the increase in RS in *b10*, but further analysis is required to investigate this possibility.

Although much more needs to be learned about the mechanisms by which RS is created in the *ssIIIa* mutant, we now know that it depends on the highly expressed *indica* Wx^a gene and involves accumulation of AAC, lipid, and amylose–lipid complex, which constitutes RS type 5 (RS 5). It is known that the SSIIIa protein is associated with other proteins in developing rice endosperm (32), and in maize a proportion of SSIIIa is also present in a large complex including ADP–glucose pyrophosphorylase (AGPase), pyruvate orthophosphate dikinase (PPDK), SSIIa, and SBEIIa and SBEIIb (33). The enzyme PPDK catalyzes a reversible reaction from pyruvate, ATP, and Pi to phosphoenolpyruvate (PEP), AMP, and PPi (34), but in photosynthesis, it operates in the direction of PEP formation, driven by the hydrolysis of PPi (34). Cereal endosperm contains both cytosolic and plastidial PPDK isoforms. The *floury endosperm-4* mutant of rice, which lacks cytosolic OsPPDKB, has smaller kernels and correspondingly less starch, but higher levels of lipid, showing that cytosolic PPDK has a role in the provision of carbon to the plastid, which in turn influences the partitioning of carbon between starch and lipid (35). A key role is also proposed for plastidial PPDK in *Zea mays*, in which the PPi generated by PPDK can be channeled directly to AGPase within the protein complex, driving the plastidial AGPase reaction in the direction of ADPGlc breakdown to Glc-1-P, which can in turn support amino acid and lipid biosynthesis (33). This close association of PPDK and plastidial AGPase may provide the means to avoid hydrolysis of PPi by a high level of pyrophosphorylase activity in the stroma. It is further proposed that the starch biosynthetic enzymes in the protein complex can exert a constraining effect on PPDK and AGPase to control the partitioning of ADPGlc into lipid or starch. It is known that uptake of ADPGlc by the plastid and the activity of the major SS enzymes do not limit carbon flux into starch, but that other constraints within the stroma control the flux into starch (36).

Based on our findings in this work and published results (32–35), we now propose a RS biosynthetic pathway (Fig. 6). We propose that loss of function of *SSIIIa* will disrupt the protein complex, consisting of PPDK, SSIIIa, AGPase, SSIIa, SBEIIa, and SBEIIb, and that this disruption will reduce the influence on PPDK and AGPase activities so that relatively more ADPGlc is directed toward glycolytic intermediates to support lipid biosynthesis via the action of pyruvate kinase (Fig. 6). Meanwhile, deficiency in SSIIIa will decrease amylopectin biosynthesis and result in a shift in carbon allocation toward amylose biosynthesis by GBSSI encoded by the *Wx* gene. Consequently, the increased levels of amylose and lipids together give rise to an increase in amylose–lipid complex, constituting RS 5. This proposed RS biosynthetic pathway could also potentially explain the formation of RS in mutants and transgenic plants with impaired expression of *SSIIa* or *SBEII* genes (15, 20).

In principle, the same *ssIIIa* mutation could be used in *japonica* rice together with introduction of a Wx^a gene, but the resulting rice would have higher amylose content than is normally preferred by consumers of *japonica* varieties. However, in the future, it may be valuable to elevate the lipid content or to pyramid the *ssIIIa* mutant with selected *Wx* alleles with intermediate levels of expression to breed new varieties with increased RS, yet with acceptable amylose content. Our discovery provides an immediate and simple way to increase RS in cooked rice, which is a staple food throughout southern Asia.

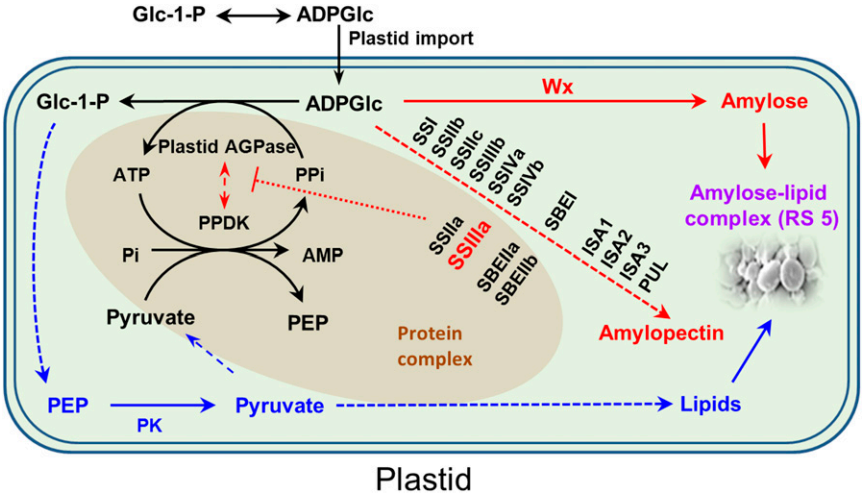

Fig. 6. A proposed RS biosynthetic pathway in the plastid. Biosynthesis of ADPGlc is brought about primarily by cytosolic AGPase, and ADPGlc is then imported into the plastid for starch biosynthesis. Several amyloplast enzymes exist in a large protein complex, which includes AGPase, PPDK, SSIIa, SSIIIa, SBEIIa, and SBEIIb, and this complex is thought to provide a means to control the partitioning of carbon between starch and lipids. The amyloplast contains a high level of pyrophosphatase, which keeps the concentration of PPi in the stroma very low. The presence of PPDK in a complex with AGPase may enable PPi to be channeled directly to AGPase for the conversion of ADPGlc to G-1-P and subsequently to lipid. The sequestering of PPDK in a protein complex may also prevent a futile cycle operating between PPDK and pyruvate kinase (PK). The starch biosynthetic enzymes in the complex are proposed to inhibit the activity of PPDK and AGPase (red dotted bar). In the absence of a functional SSIIIa protein, the complex is disrupted, and the influence on PPDK and AGPase is reduced, such that more carbon is directed from ADPGlc to Glc-1-P and into lipid. At the same time, the absence of SSIIIa means that relatively more ADPGlc can also be consumed by the Wx protein in the biosynthesis of amylose. This process leads to an increase in amount of amylose–lipid complex and hence RS 5. Dashed arrows indicate multiple steps. AGPase, ADP glucose pyrophosphorylase; ISA, isoamylase; PEP; phosphoenolpyruvate; PUL, pullulanase.

Materials and Methods

Plant growth, map-based cloning, qRT-PCR, plasmid construction and transformation, X-ray diffraction of starch, and chain-length distribution of amylopectin were carried out as described (37–43). RS, AAC, total starch and lipid contents, pasting properties, and microscopic features of starch granules were measured by using mature seeds. Details of experimental methods are provided in *SI Materials and Methods*. Primers used in this study are listed in Table S1.

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