Amyloplast Membrane Protein SUBSTANDARD STARCH GRAIN6 Controls Starch Grain Size in Rice Endosperm

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Starch is a biologically and commercially important polymer of glucose. Starch is organized into starch grains (SGs) inside amyloplasts. The SG size differs depending on the plant species and is one of the most important factors for industrial applications of starch. There is limited information on genetic factors regulating SG sizes. In this study, we report the rice (Oryza sativa) mutant substendant starch grains (ssg6), which develops enlarged SGs in endosperm. Enlarged SGs are observed starting at 3 d after flowering. During endosperm development, a number of smaller SGs appear and coexist with enlarged SGs in the same cells. The ssg6 mutation also affects SG morphologies in pollen. The SSG6 gene was identified by map-based cloning and microarray analysis. SSG6 encodes a protein homologous to aminotransferase. SSG6 differs from other rice homologs in that it has a transmembrane domain. SSG6-green fluorescent protein is localized in the amyloplast membrane surrounding SGs in rice endosperm, pollen, and pericarp. The results of this study suggest that SSG6 is a novel protein that controls SG size. SSG6 will be a useful molecular tool for future starch breeding and applications.

Therefore, the amyloplast volume is considered as approximately equivalent to the SG volume (Matsushima et al., 2014). Rice (Oryza sativa) endosperm SGs are normally 10 to 20 μm in diameter (Matsushima et al., 2010). Each amyloplast contains a single SG that is organized from the assembly of several dozen smaller starch granules. Each starch granule is a sharp-edged polyhedron with a typical diameter of 3 to 8 μm. This type of SG is called a compound SG (Tateoka, 1962). For compound SGs, starch granules are assembled but not fused to form a single SG, which is easily separated by conventional purification procedures. By contrast, a simple SG contains a single starch granule (Tateoka, 1962). In this case, both terms are used equally. Simple SGs are produced in several important crops such as maize (Zea mays), sorghum (Sorghum bicolor), barley (Hordeum vulgare), and wheat (Triticum aestivum; Tateoka, 1962; Matsushima et al., 2010, 2013). SG sizes in cereal endosperm are diverse. Maize and sorghum SGs have a uniform size distribution of approximately 10 μm in diameter (Jane et al., 1994; Matsushima et al., 2010; Ai et al., 2011). In barley and wheat, SGs of two discrete size classes (approximately 15–25 μm and less than 10 μm) coexist in the same cells (Evers, 1973; French, 1984; Jane et al., 1994; Matsushima et al., 2010, 2013).

The size of starch granules is one of the most important characteristics for industrial applications (Lindeboom et al., 2004). Small starch granules can replace fat in food applications, because aqueous dispersions of small

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starch granules show fat-mimetic properties (Malinski et al., 2003). The larger starch granules of maize and cassava (Manihot esculenta) improve the final starch yield after wet-milling purification (Gutiérrez et al., 2002). In the case of simple SGs, the size of SGs is equal to the size of starch granules. Therefore, manipulation of the sizes of SGs and starch granules is a molecular target for bioengineering programs. SG size can be reduced in transgenic plants and genetic mutants by down-regulating several starch synthetic enzymes (Gutiérrez et al., 2002; Bustos et al., 2004; Ji et al., 2004; Stahl et al., 2004; Matsushima et al., 2010; Fujita, 2014). By contrast, our understanding of the genetic tools, biosynthetic enzymes, and plant materials that can be utilized to enlarge SGs is limited.

Recent work identified a rice mutant that develops enlarged SGs; this mutant has been named substandard starch grain4 (ssg4; Matsushima et al., 2014). The enlarged SGs are observed in starch-accumulating tissues of ssg4, including endosperm, pollen, root caps,

**Figure 1.** Enlarged SGs of mature ssg6 endosperm. A, Wild-type cv Koshihikari (WT) seeds. Bar = 1 mm. B, ssg6 seeds. Bar = 1 mm. C, Quantification of wild-type and ssg6 seed sizes (n = 30 each). D, One-hundred seed weight of the wild type and ssg6 (n = 16 and 15, respectively). E and G, Iodine-stained thin sections of wild-type endosperm at low and high magnification, respectively. Bars = 10 μm. F and H, Iodine-stained thin sections of ssg6 endosperm at low and high magnification, respectively. Bars = 10 μm. I, Quantification of the areas occupied by SGs in sections of different genotypes (n ≥ 30 each). J, Quantification of the starch amount in mature seeds expressed as the percentage of seed weight (n = 6 each). Data are given as means ± so. Statistical comparisons were performed using Welch’s t test; all data were compared with the wild type (**, P < 0.01; *, P < 0.05; and ns, not significant at P = 0.05).

**Table 1.** Effects of the ssg6 mutation on gelatinization properties of endosperm starch determined by differential scanning calorimetry

Gelatinization properties of starch in ssg6 seeds were analyzed by differential scanning calorimetry. Values are means ± so of nine independent determinations. T_o, T_p, and T_c are onset, peak, and conclusion temperatures, respectively. ΔH is gelatinization enthalpy of starch. Asterisks indicate significance at P < 0.01.

<table>
<thead>
<tr>
<th>Plant</th>
<th>T_o (°C)</th>
<th>T_p (°C)</th>
<th>T_c (°C)</th>
<th>ΔH (mJ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type cv Koshihikari</td>
<td>54.9 ± 0.7</td>
<td>63.9 ± 0.6</td>
<td>71.2 ± 0.2</td>
<td>8.6 ± 1.3</td>
</tr>
<tr>
<td>ssg6</td>
<td>56.3 ± 1.7</td>
<td>65.8 ± 0.9</td>
<td>72.9 ± 1.0</td>
<td>9.3 ± 1.1</td>
</tr>
</tbody>
</table>

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and young pericarp. Chloroplasts in young ssg4 leaves also are enlarged. SSG4 encodes an amyloplast-localized large protein with a domain of unknown function (DUF490). SSG4 homologs are conserved from bacteria to higher plants; however, the exact molecular function of SSG4 is unknown.

In this study, we report the identification of another rice mutant (ssg6) that develops enlarged SGs in endosperm. We characterize the ssg6 mutation and identify the responsible gene. SSG6 encodes a protein homologous to aminotransferase. SSG6 is localized at the amyloplast membrane and influences SG size. We also determined that ssg4 and ssg6 mutations act synergistically in pollen. SSG6 will be a useful molecular target for future starch breeding and starch biotechnology programs.

Figure 2. Developing SGs in maturing endosperm. A to C, Wild-type cv Koshihikari (WT) seeds at 3, 5, and 7 DAF, respectively. Bars = 1 mm. D to F, ssg6 seeds at 3, 5, and 7 DAF, respectively. Bars = 1 mm. G to I, Iodine-stained thin sections of wild-type endosperm at 3, 5, and 7 DAF, respectively. Bars = 20 μm. J to L, Iodine-stained thin sections of ssg6 endosperm at 3, 5, and 7 DAF, respectively. Bars = 20 μm. M, Quantification of the areas occupied by SGs at 3, 5, and 7 DAF (n = 11 each). N and O, Higher magnifications of iodine-stained thin sections of wild-type and ssg6 endosperm at 7 DAF, respectively. Bars = 10 μm. P, Kernel density plot for the areas occupied by SGs at 7 DAF. The wild type and ssg6 are indicated by red and black lines, respectively (n = 300 and 299, respectively). Q, Quantification of the numbers of SGs per 5,833 μm² at 7 DAF. Data are given as means ± SE. Statistical comparisons were performed by Welch’s t test; all data were compared with the wild type (**, P < 0.01; and ns, not significant at P = 0.05).
Figure 3. SGs in pollen and chloroplasts in third leaves. A and B, Iodine-stained pollen of wild-type cv Koshihikari (WT) and ssg6, respectively. Bars = 10 μm. C and D, Released SGs from squashed pollen of the wild type and ssg6, respectively. Bars = 10 μm. E, Quantification of the areas occupied by SGs in pollen (n = 40 each). F and G, Third leaves of the wild type and ssg6, respectively. Bars = 1 mm. H and I, Thin sections of the third leaves of the wild type and ssg6 were double stained with Methylene Blue and Basic Fuchsin. Bars = 10 μm. J and K, Magnified images of H and I. Bars = 10 μm. L, Quantification of the areas occupied by chloroplasts in third leaves (n = 16 each). The quantification was based on three and five TEM sections of the wild type and ssg6, respectively. Data are given as means ± SD. Statistical comparisons were performed by Welch’s t test (**, P < 0.01). M and N, TEM images of chloroplasts of the wild type and ssg6, respectively. Bars = 1 μm.
RESULTS

Enlarged SGs in Mature ssg6 Endosperm

We previously developed a rapid method to observe SGs in rice endosperm (Matsushima et al., 2010). Using this method, we performed a genetic screen of a mutagenized cv Nipponbare population and isolated mutants with defective SGs (Matsushima et al., 2010). We repeated these experiments using cv Koshihikari, which is an elite variety in Japan. In this study, we performed the same genetic screen using a mutagenized cv Koshihikari population and isolated ssg6. The ssg6 seed morphology was slightly chalky compared with wild-type cv Koshihikari seeds (Fig. 1, A and B). Polished ssg6 grains were chalky (Supplemental Fig. S1A). The ssg6 grain size was slightly smaller than that of the wild type, especially with respect to grain width and depth (Fig. 1C). The ssg6 grain weight was less than that of the wild type (Fig. 1D). Iodine-stained thin sections of mature ssg6 endosperm clearly showed enlarged SGs (Fig. 1, E–H). Quantification of SG areas in thin sections showed that ssg6 SGs were more than 2 times larger than those in the wild type (Fig. 1I). Next, we quantified the number and size of starch granules in SGs of mature endosperm. The number of starch granules was increased in enlarged SGs of ssg6 compared with wild-type SGs (Supplemental Fig. S1B). In contrast, the size of starch granules decreased slightly in ssg6 (Supplemental Fig. S1C). Therefore, the increased size of ssg6 SGs was mainly attributed by the increased numbers of starch granules.

The formation of SGs in developing endosperm has four possible genotypes at one gene locus (i.e. AAA, AAs, Aat, and aat). We performed reciprocal crosses to obtain two distinct heterozygous seeds of SSG6SSG6ssg6 and SSG6ssg6ssg6. The SG sizes of heterozygous seeds and wild-type seeds did not differ significantly (Fig. 1I). This indicated that one copy of the wild-type allele supplied from the male gametophyte was sufficient for the formation of normal-sized SGs.

We examined starch accumulation in ssg6 seeds. The total amount of starch as a percentage of weight was not significantly different between ssg6 and wild-type seeds (Fig. 1J). Amylopectin chain-length distribution and amylopectin gelatinization temperature were similar in ssg6 and wild-type starch (Table I; Supplemental Fig. S1D). The amylose content was slightly lower in ssg6 starch than in wild-type starch (Supplemental Fig. S1E). We analyzed soluble sugars in ssg6 and wild-type seeds using gas chromatography-time-of-flight-mass spectrometry. Glc, Fru, and Suc levels were significantly higher in ssg6 than in wild-type seeds (Supplemental Fig. S2). Man, trehalose, Xyl, and raffinose levels were not significantly different in ssg6 and wild-type seeds (Supplemental Fig. S2).

Dynamic Behavior of SGs in Developing Endosperm

We investigated when enlarged SGs developed in the ssg6 mutant by analyzing developing seeds at 3, 5, and 7 d after flowering (DAF). From 3 to 7 DAF, seed enlargement was essentially the same in ssg6 and the wild type (Fig. 2, A–F), but SG sizes differed (Fig. 2, G–L). At 3 DAF, most SGs in ssg6 endosperm were larger than those in the wild type (Fig. 2, G and J).

We also used transmission electron microscopy (TEM) to assess ssg6 endosperm at 3 DAF (Supplemental Fig. S3). The ssg6 SG morphologies in TEM images were spherical and large, similar to the iodine-stained SGs. The ssg6 enlarged SGs occupied an area more than 10-fold larger than that of normal-sized wild-type SGs (Fig. 2M). At 7 DAF, the area occupied by SGs was more than 2.5-fold larger in ssg6 than in the wild type (Fig. 2M). The quantification does not include the very small SGs, in order to compare only the enlarged SGs. Meanwhile, the number of small SGs increased and coexisted with the enlarged SGs in the same cells during endosperm development in ssg6.

At 7 DAF, the outlines of both small and enlarged SGs were sharp (Fig. 2, N and O). Therefore, we quantified the sizes of both small and enlarged SGs in 7-DAF endosperm. At 7 DAF, wild-type endosperm contained SGs with uniform sizes (Fig. 2N). By contrast, ssg6 endosperm contained a number of smaller SGs in addition to the enlarged SGs (Fig. 2O). Kernel density estimation indicated that ssg6 contained primarily small SGs less than 50 μm² (Fig. 2P). This estimation was determined using two-dimensional sections; therefore, the method may underestimate SG sizes and overestimate small SG densities due to SGs oriented in a nonsagittal plane. However, small SGs appear to be more abundant in ssg6 than in the wild type (Fig. 2, N and O).

In ssg6 endosperm, enlarged SGs with areas greater than 400 μm² were not as abundant as small SGs. In wild-type endosperm, the majority of SGs had sizes of 100 to 200 μm². The total numbers of SGs per area were approximately the same in ssg6 and the wild type (Fig. 2Q). At 3 and 5 DAF, the small SGs of ssg6 are spherical and large, similar to the iodine-stained SGs.

Table II. Segregation of ssg6 pollen phenotypes in F1 plants

<table>
<thead>
<tr>
<th>Parental Genotype</th>
<th>No. of Wild-Type Pollen</th>
<th>No. of ssg6 Pollen</th>
<th>Total</th>
<th>Percentage of ssg6 Pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssg6+/−</td>
<td>31</td>
<td>24</td>
<td>55</td>
<td>43.6</td>
</tr>
</tbody>
</table>

Table III. Segregation of ssg6 seeds in the F2 population

<table>
<thead>
<tr>
<th>Parental Genotype</th>
<th>No. of ssg6 Seeds</th>
<th>No. of Wild-Type Seeds</th>
<th>Total</th>
<th>χ² Value (P Value) for 1:3 Segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssg6+/−</td>
<td>30</td>
<td>99</td>
<td>129</td>
<td>0.21 (0.65)</td>
</tr>
</tbody>
</table>
were not stained clearly with iodine and did not have clear outlines. That made it difficult to quantify their sizes.

We also investigated the protein accumulation of starch biosynthetic enzymes in developing seeds (Supplemental Fig. S4). Accumulation levels of starch synthases, branching enzymes, phosphorylase, and pullulanase did not change between wild-type and ss6 developing seeds.

**Pollen SGs and Leaf Chloroplasts**

Pollen, root cap, and pericarp also accumulate high levels of starch in rice. The ss6 pollen developed spherical SGs, whereas wild-type pollen developed rod-like SGs (Fig. 3, A–D). Areas occupied by SGs were slightly less in ss6 than in the wild type (Fig. 3E). When ss6 was crossed with the wild type, approximately half of the F1 pollen displayed ss6 phenotypes (Table II). This indicates that ss6 behaves in a gametophytic manner in pollen. The SGs in root cap and pericarp were similar in ss6 and the wild type (Supplemental Fig. S5).

A variegated phenotype was observed in the third leaves of ss6 mutants (Fig. 3, F and G). Therefore, we speculated that chloroplasts were affected by the ss6 mutation. To visualize chloroplasts, thin sections of third leaves from young plants were double stained with Methylene Blue and Basic Fuchsin (Fig. 3, H–K). Wild-type chloroplasts displayed lens-like shapes, whereas ss6 chloroplasts were more spherical.
Chloroplast areas were approximately 3-fold larger in ssg6 than in the wild type (Fig. 3L). These results indicate that the ssg6 mutation affects chloroplast size. We used TEM to observe ssg6 chloroplast ultrastructures, starch granules, and grana stacks. The results showed that starch granule sizes and grana stacks were similar in ssg6 and wild-type chloroplasts (Fig. 3, M and N). Based on the TEM images, we quantified the number of chloroplasts per cell and the number of starch granules per chloroplast (Supplemental Fig. S6, A and B). In the ssg6 third leaves, the number of chloroplasts in one cell were decreased compared with the wild type (Supplemental Fig. S6A). The enlarged chloroplasts in ssg6 third leaves tend to contain many more starch granules compared with the wild-type chloroplasts (Supplemental Fig. S6B). This is consistent with the previous observation that enlarged chloroplasts in Arabidopsis (*Arabidopsis thaliana*) plastid division mutants contained increased numbers of starch granules (Crumpton-Taylor et al., 2012).

The ssg6 flag leaves were not variegated (Supplemental Fig. S7, Å and B). The flag leaf chloroplasts were slightly larger in ssg6 than in the wild type but did not differ to the extent of chloroplasts in the third leaves of ssg6 and the wild type (Supplemental Fig. S7, C–G). TEM analysis of chloroplast ultrastructure in flag leaves indicated that there were no significant differences between ssg6 and the wild type (Supplemental Fig. S7, H and I). As in the third leaf, the number of chloroplasts per cell was decreased and the number of starch granules per chloroplast was increased in ssg6 flag leaves (Supplemental Fig. S6, C and D).

### Identification of the SSG6 Gene

The ssg6 phenotype in endosperm tissue was completely penetrant in ssg6-selfed progeny and segregated as a single recessive allele in F2 progeny (Table III). We identified the SSG6 gene using conventional map-based cloning. We mapped the ssg6 mutation within a 152-kb region on chromosome 6 (Fig. 4A). Twenty-nine open reading frames were expected in this region according to the Rice Annotation Project (RAP) database (http://rapdb.dna.affrc.go.jp/). To narrow down the candidate genes, we performed DNA microarray analysis to investigate gene expression in 4-DAF seeds. We expected the expression of the mutated gene to be reduced in ssg6 mutants. Out of the 29 candidate genes, 20 genes had at least one probe on the Agilent Rice Oligo DNA Microarray 44K that we used. Of these, a single probe (Os06g0130400|mRNA|AK065212|CDS+3’UTR) showed significantly reduced signal intensity in the ssg6 mutant compared with that of the wild type (Supplemental Fig. S8). This indicated that Os06g0130400 gene expression was reduced in ssg6.

Next, we determined the nucleotide sequence of Os06g0130400 in ssg6 and the wild type. We identified a base change in the Os06g0130400 gene of ssg6 (Supplemental Fig. S9); the mutant carried a C-to-T transition at position 1,630,075 (Os-Nipponbare-Reference-IRGSP-1.0-based position). The C-to-T transition was consistent with an ethyl methanesulfonate-induced mutation. Os06g0130400 encoded a protein containing 542 amino acid residues (Fig. 4B). The mutation in ssg6 introduced a premature stop codon at amino acid position 63 (Fig. 4B; Supplemental Fig. S9). The Os06g0130400 protein was predicted to have an...
aminotransferase class I and II domain (Aminotran_1_2 domain) according to the Pfam database (Fig. 4B; Supplemental Fig. S9).

We performed a complementation test using the maize UBIQUITIN1 promoter to express Os06g0130400 cDNA. The cDNA sequence was confirmed by 5’ and 3’ RACE experiments. The cDNA clone was introduced into the ssg6 mutant, and transgenic plants homozygous for the transgene were isolated and named UBi:SSG6cDNA/ ssg6. SG sizes and morphologies in UBi:SSG6cDNA/ ssge6 endosperm and pollen were very similar to those in the wild type (Fig. 5). This indicates that SG phenotypes in endosperm and pollen were completely rescued by the transgene. We conclude that Os06g0130400 is the gene responsible for the ssg6 mutation.

SSG6 (Os06g0130400) shows homology with 1-aminocyclopropane-1-carboxylate synthase (ACS) and was named OsACS6 according to the RAP database. Arabidopsis and rice genomes contain 12 and six annotated ACS genes, respectively (Yamagami et al., 2003; Iwai et al., 2006). In Arabidopsis, ACS1 was enzymatically inactive and ACS3 was a pseudogene, whereas ACS2, ACS4, ACS5, ACS6, ACS7, ACS8, ACS9, and ACS11 encode enzymatically active ACS proteins (Yamagami et al., 2003). ACS10 and ACS12 were shown to have aminotransferase but not ACS activity (Yamagami et al., 2003). SSG6 (OsACS6) was phylogenetically related to ACS10 and ACS12 (Fig. 4C; Supplemental Fig. S10). In rice, other ACS homologs (OsACS1–OsACS5) belonged to ACS.

The transmembrane prediction program TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) predicted one transmembrane domain in the SSG6 protein (Fig. 4D). One transmembrane domain also was predicted for Arabidopsis ACS10 and ACS12 (Fig. 4D). By contrast, no transmembrane domains were predicted in rice ACSs (OsACS1–OsACS5).

Subcellular Localization of the SSG6 Protein

SSG6 contains one putative transmembrane domain (Fig. 4D). Therefore, SSG6 may be localized to an intracellular membrane. To analyze the subcellular localization of SSG6-GFP in various tissues, confocal and differential interference contrast (DIC) images of SSG6-GFP transgenic plants are shown. A to C, Whole-mount images of pollen. Bar = 5 μm. D to F, Higher magnification images of amyloplasts in pollen. Bar = 5 μm. G to I, Endosperm sections obtained by vibratome sectioning. Bar = 5 μm. J to L, Pericarp sections obtained by vibratome sectioning. Bar = 5 μm.

localization of SSG6, we generated a construct containing the full-length SSG6 cDNA fused to GFP (SSG6-GFP). SSG6-GFP was transiently expressed in Nicotiana benthamiana leaves, and SSG6-GFP fluorescence was detected surrounding chlorophyll autofluorescence (Supplemental Fig. S11). This suggests that SSG6 is localized at the chloroplast envelope membrane.

We generated stable transgenic rice plants expressing SSG6-GFP under the control of the maize UBIQUITIN1 promoter. In transgenic SSG6-GFP rice plants, SSG6-GFP fluorescence was detected in pollen, endosperm, and pericarp (Fig. 6). In pollen, SSG6-GFP fluorescence was observed as numerous small ring-like structures (Fig. 6, A–F). Differential interference contrast images of pollen showed that the ring-like GFP fluorescence surrounded rod-shaped structures (Fig. 6, E and F); these structures are likely to be SGs, because their morphologies are consistent with the iodine-stained SGs of wild-type pollen (Fig. 3, A and C). Pollen SGs surrounded by SSG6-GFP were composed of a single starch granule (Fig. 3, A–F). This means that SGs in pollen were simple SGs.

In developing endosperm and pericarp, SSG6-GFP fluorescence was detected at the membrane of amyloplasts, which contained compound SGs (Fig. 6, G–L). Taken together, these data suggest that SSG6 is localized at the amyloplast membrane in several tissues.

**Agronomic Traits of ssg6**

To examine whether the ssg6 mutation affects rice growth under natural conditions, we grew the third-backcross generation (BC3F2) of ssg6 and wild-type plants in a paddy field and compared major agronomic traits, including duration to heading, culm length, panicle length, total panicle number, number of spikelets per panicle, number of sterile spikelets per panicle, and total panicle weight. Under natural conditions, whole-plant phenotypes did not differ significantly between BC3F2 ssg6 and the wild type (Fig. 7A).

Of the seven agronomic traits measured, four traits showed statistically significant differences between BC3F2 ssg6 and the wild type (Fig. 7, B–H). The duration to heading, culm length, and number of panicles were reduced slightly in BC3F2 ssg6 compared with those in the wild type. Total panicle weight also was lower in BC3F2 ssg6 than in the wild type; this is considered to be due to the reduced number of total panicles and a slight reduction of seed weight in BC3F2 ssg6 compared with the wild type (Fig. 1D).

**SGs in Pollens of ssg4 ssg6 Double Mutants**

sgs4 and ssg6 mutants had similar phenotypes: enlarged SGs in endosperms and spherical SGs in pollens.

**Table IV. Segregation of the selfed progeny of ssg4+/ssg6+**

<table>
<thead>
<tr>
<th>Parental Genotype</th>
<th>No. of ssg4+/ssg6+</th>
<th>No. of ssg4−/ssg6−</th>
<th>No. of ssg4−/ssg6−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssg4−/ssg6−</td>
<td>0</td>
<td>88</td>
<td>93</td>
<td>181</td>
</tr>
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</table>
We tried to construct ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> double mutant to investigate how the two mutations interact. In the F2 populations between ssg4 and ssg6, ssg4<sup>−/−</sup> ssg6<sup>−/−</sup> and ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> mutants were obtained. The selfed progeny of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> and ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> plants were genotyped at the ssg4 and ssg6 loci (Tables IV and V). Out of 181 progeny of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> and 339 progeny of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup>, no double homozygous mutant (ssg4<sup>−/−</sup>ssg6<sup>−/−</sup>) was obtained. This raised the possibility that the transmission of male and female gametophytes was affected by the double mutations. We checked the transmission efficiencies of male and female gametophytes of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> and ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> by reciprocal crosses with cv Koshihikari (Tables VI and VII). In both ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> and ssg4<sup>−/−</sup>ssg6<sup>−/−</sup>, the transmission of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> male gametophytes was significantly impaired. In contrast, the transmission of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> female gametophytes was not affected. This suggested that male gametophytes of the ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> double mutant reduced activity compared with the ssg4 and ssg6 single mutant gametophytes.

Iodine-stained pollens obtained from ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> and ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> plants were segregated with respect to the SG morphologies (Fig. 8). Some pollens contained spherical simple SGs like ssg4 and ssg6 single mutants (Fig. 8, A and B). Other pollens showed enlarged compound SGs (Fig. 8, C and D). TEM images confirmed that the spherical SGs were composed of a single starch granule; in contrast, the enlarged SGs were assemblies of starch granules (Fig. 8, E–H). The segregation of pollens with simple and compound SGs was approximately 1:1 in both ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> and ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> plants (Fig. 8I).

### DISCUSSION

#### Regulation of SG Size by SSG6

In this study, we used our rapid method to observe SGs in rice endosperm and isolated the ssg6 mutant from a population of mutagenized cv Koshihikari rice. SGs in ssg6 endosperm were enlarged, similar to those in the ssg4 mutant (Fig. 1, E–I; Matsushima et al., 2014). SGs in ssg6 pollens are sphere shaped, similar to those in the ssg4 mutant (Fig. 3, A–D; Matsushima et al., 2014). By contrast, SGs in ssg6 root cap and pericarp are similar to those of the wild type but differ from those in the ssg4 mutant (Supplemental Fig. S5; Matsushima et al., 2014). This indicates that SG size is controlled by SSG6 in all tissues, whereas the requirement for SSG4 activity in regulating SG size varies in different tissues.

The ssg6 mutation introduces a premature stop codon into the coding region, which generates a truncated protein whose length is approximately 90% shorter than that of the wild type (Fig. 4B; Supplemental Fig. S9). The missing region contains part of the transmembrane domain and all of the Aminotran_1_2 domain. Therefore, the ssg6 mutation is probably a null mutation, although ssg6 phenotypes appear to be mild. The seeds of ssg4 were more chalky than those of ssg6 (Fig. 1, A and B; Supplemental Fig. S1A; Matsushima et al., 2014), and not all endosperm SGs were enlarged in ssg6 (Fig. 2, N–P). During endosperm development in ssg6, the number of smaller SGs increased and coexisted with enlarged SGs in the same cells (Fig. 2, G–L). By contrast, an increase in smaller SGs was not obvious in ssg4 developing endosperm (Matsushima et al., 2014). The smaller SGs may compensate for the starch amount in ssg6. Consistent with this, the total starch amount in ssg6 endosperm did not change during development (Fig. 1J), whereas the total starch amount in ssg4 endosperm decreased during development (Matsushima et al., 2014).

The coexistence of small and large SGs in the same cells also was observed in barley and wheat cereal crops (Evers, 1973; French, 1984; Jane et al., 1994; Matsushima et al., 2010). This is called bimodal SGs. A phylogenetic analysis of Poaceae shows that only five genera contain species that develop bimodal SGs: Brachypodium, Triticum, Hordeum, Elymus, and Bromus (Tateoka, 1962; Matsushima et al., 2013). This suggests that bimodal SGs are phylogenetically specific for Poaceae. The molecular mechanisms that regulate natural variations in SG morphologies are currently unknown. The activities of SSG6 homologs in these cereal crops may be involved in this natural morphological variation.

The size of the starch granule is a target for breeding and biotechnology programs to improve starch uses in industrial applications (Lindeboom et al., 2004). However, the enlargement of SGs in ssg6 does not result in the direct expansion of starch granules (Supplemental Fig. S1C). SGs in ssg6 are compound SGs that separate into starch granules during processing. Therefore, the

### Table V. Segregation of the selfed progeny of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of ssg4&lt;sup&gt;−/−&lt;/sup&gt;ssg6&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssg4&lt;sup&gt;−/−&lt;/sup&gt;ssg6&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>175</td>
<td>339</td>
</tr>
</tbody>
</table>

### Table VI. Genetic transmission analysis of the ssg4<sup>−</sup>ssg6<sup>−</sup> mutation

<table>
<thead>
<tr>
<th>Parental Cross</th>
<th>No. of ssg4&lt;sup&gt;−/−&lt;/sup&gt;ssg6&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>cv Koshihikari × ssg4&lt;sup&gt;−/−&lt;/sup&gt;ssg6&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>58</td>
<td>3.4%</td>
</tr>
<tr>
<td>ssg4&lt;sup&gt;−/−&lt;/sup&gt;ssg6&lt;sup&gt;−/−&lt;/sup&gt; × cv Koshihikari</td>
<td>16</td>
<td>69.6%</td>
</tr>
</tbody>
</table>

<sup>aTE (transmission efficiency) = number of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup>/number of ssg4<sup>−/−</sup>ssg6<sup>−/−</sup> × 100.</sup>
ssg6 mutant is not the direct target of breeding programs. In contrast, in the case of simple SGs, the size of SGs is industrially important, because simple SGs do not separate into starch granules and the size of simple SGs is equal to that of the starch granule. Therefore, SSG6 homolog mutants of other crops that develop simple SGs, such as maize, sorghum, and barley, will achieve engineered starch granule enlargement.

Subcellular Localization of SSG6

SSG6-GFP fluorescence is detected on the envelope membrane of amyloplasts and chloroplasts (Fig. 6; Supplemental Fig. S11). This suggests that SSG6 is a membrane protein of these organelles. ACS12 also is localized at the chloroplast envelope membrane, according to the AT_CHLORO database (http://www.grenoble.prabi.fr/at_chloro/), which collates proteomics information for intraplastidic protein localization in Arabidopsis (Ferro et al., 2010). The putative transmembrane domains of SSG6 and ACS12 should be functional.

The membrane topology and subplastid localization of SSG6 protein are crucial to understand SSG6 function. Experiments to obtain such information are necessary in the future.

Possible Functions of the SSG6 Protein

ACS catalyzes the conversion of S-adenosyl-Met to 1-aminocyclopropane-1-carboxylic acid, which is the precursor of ethylene. This reaction is the rate-limiting step in the ethylene biosynthetic pathway (Bleecker and Kende, 2000). ACS isozymes are well studied (Yamagami et al., 2003; Tsuchisaka et al., 2009). Arabidopsis ACS10 and ACS12 were reported to not have ACS activity; therefore, these proteins have been largely excluded from previous analyses (Yamagami et al., 2003; Tsuchisaka et al., 2009). Here, we show that SSG6 has highest homology with ACS10 and ACS12 (Fig. 4C). SSG6, ACS10, and ACS12 contain putative transmembrane domains, whereas no other ACSs contained these domains (Fig. 4D). Therefore, ACS10 and ACS12 are functional homologs of SSG6 in Arabidopsis. ACS10 and ACS12 were shown to be aminotransferases (Yamagami et al., 2003). Therefore, SSG6 also may have aminotransferase activity.

OsAlaAT1 is a cytosolic aminotransferase that is responsible for the conversion of pyruvate to Ala in rice.
endosperm (Yang et al., 2015). The osalaat1 mutation decreased the expression of starch biosynthetic genes and generated floury endosperm. Pyruvate-related metabolism was suggested to affect the substrate supply for starch synthesis (Yang et al., 2015). Further characterization of SSG6 will uncover the unknown connection between aminotransferase and starch metabolism.

The rice expression profile database RiceXPro (http://ricexpro.dna.affrc.go.jp/) indicates that SSG6 is expressed ubiquitously in nonstorage and storage organs (Sato et al., 2011). A variegated phenotype was observed in the third leaves of ssg6 mutants (Fig. 3, F and G). Chloroplasts were enlarged in third and flag leaves of ssg6 (Fig. 3, H–N; Supplemental Fig. S7). Therefore, SSG6 has a role in regulating chloroplast size in non-storage organs. This may be related to the slight reduction in duration to heading, culm length, and number of panicles in ssg6 grown in a paddy field (Fig. 7). Neither acs10 nor acs12 mutants have been analyzed. Phenotypic analysis of those mutants will clarify the biological functions of these gene groups in vivo.

**Interaction of ssg4 and ssg6 Mutations**

Half of the pollens of ssg4−/−ssg6−/− and ssg4+/−ssg6−/− showed severe phenotypes with enlarged compound SGs (Fig. 8f). Because the other half of pollens contained spherical simple SGs like ssg4 and ssg6 single mutant pollens, pollens with the enlarged compound SGs will be in the ssg4−/−ssg6−/− genotype. This means that ssg4 and ssg6 mutations act synergistically in the size control of pollen SGs. The ssg4−ssg6− pollens should develop normally but have severely reduced activity of transmission. That can explain the segregation distortion of selfed progeny of ssg4−/−ssg6−/− and ssg4+/−ssg6−/− (Tables IV and V). However, we could not exclude the possibility that postfertilization lethality, such as embryo lethality, contributes to the unavailability of the double homozygous mutant (ssg4−/−ssg6−/−), because the male transmission of ssg4−ssg6− was not zero (Tables VI and VII). An Arabidopsis knockout mutant of the SSG4 homolog (At2g25660) showed embryo lethality (Meinke et al., 2008). It is possible that the synergistic effect of ssg4 and ssg6 mutations may occur in other tissues in addition to the pollens.

Amyloplasts and chloroplasts both develop from proplastids (Sakamoto et al., 2008). Chloroplast size is controlled by the chloroplast binary fission division machinery, especially by the ring structures at the division sites (Miyagishima, 2011). Rice mutants defective in the ring structures develop enlarged and dumbbell-shaped chloroplasts in leaves, but they do not develop enlarged amyloplasts (Yun and Kawagoe, 2009; Kamau et al., 2015). Therefore, ssg4 and ssg6 mutants are unique in that they affect both chloroplasts and amyloplasts. The mechanism to develop compound SGs in ssg4 ssg6− pollens was unknown. Proplastids before starch accumulation may be already enlarged in ssg4−ssg6− pollens and retain multiple priming sites of starch granule synthesis. We believe that ssg4 and ssg6 mutants are useful for future starch breeding programs and for basic understanding of the mechanisms regulating plastid size in plants.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Rice (Oryza sativa) ssp. japonica ‘Koshihikari’ and ‘Nipponbare’ and ssp. indica ‘Kasalath’ were used as wild-type plants. The ssg6 mutant was isolated from a mutagenized cv Koshihikari population. Mutagenization was carried out by soaking seeds in 1.5% (v/v) methanesulfonic acid ethyl ester (Nacalai Tesque; 15159–54). The M2 lines derived from a single M1 plant were grown, and M2 seeds were collected from individual M1 plants after self-fertilization. We backcrossed the ssg6 mutant with the wild type, and their progeny with ssg6 phenotypes were used in this study. The ssg6 mutant was also backcrossed with cv Nipponbare for transformation of complementation test. Rice plants were grown at an experimental paddy field of the Institute of Plant Science and Resources, Okayama University, under natural conditions. Transgenic plants were grown at 28°C in a greenhouse.

**Isolation of ssg6 by the Rapid Observation Method**

Screening was carried out with at least five seeds from each M2 line. Endosperm thin sections from M2 seeds were prepared, and SGs were observed by the previously developed method (Matsushima et al., 2010).

**Characterization of Grain Appearance, Sizes, and Starch Amounts**

 Matured dry seeds and maturing young seeds were photographed with a macromicroscope (MVX10; Olympus) and a digital camera (D772; Olympus). The sizes of grains were measured with a Vernier caliper. The total amount of starch was measured by enzymatic methods using the Total Starch Assay Kit (Megazyme International).

**Thermal Properties of Starch**

Thermal properties of starch were analyzed using a differential scanning calorimeter (DSC-6100; Seiko Instruments). The procedures were the same as in our previous study (Matsushima et al., 2014).

**Chain-Length Distribution of Amylopectin**

The chain-length distribution of amylopectin was analyzed using fluorescence labeling and capillary electrophoresis (Beckman Coulter). The procedures were the same as in our previous study (Matsushima et al., 2010).

**Semiquantification of Sugars in Mature Seeds**

After separating husks from seeds, the brown rice (50 seeds) of cv Koshihikari and the ssg6 mutant were bulked separately and then crushed using a Retsch mixer mill (MM301) at a frequency of 20 Hz for 2 min at 4°C. We prepared five pooled samples for each genotype. Extracts from the bulked seeds (equivalent to 20 mg) of each sample were subjected to gas chromatography-time-of-flight mass spectrometry as described previously (Kusano et al., 2007). Peaks of each sugar in each analyte were identified by comparing retention indices and the mass spectra of the corresponding authentic standards.

**Observation of SGs and Chloroplasts by Thin-Section Microscopy with Technovit 7100 Resin**

For matured dry seeds, approximately 1-mm cubic blocks were cut from the center region of the endosperm and fixed in a solution containing 5% (v/v) formalin, 5% (v/v) acetic acid, and 50% (v/v) ethanol for at least 12 h at room temperature.
Rice Mutant with Enlarged Starch Grains

Plasmid Construction

Because the regeneration efficiency of cv Koshihikari is lower than that of cv Nipponbare, the ss6 mutant was backcrossed with cv Nipponbare to change the genetic background. For complementation, the Os01g0179400 cDNA fragment was amplified from the full-length cDNA clone (AK065212, National Institute of Agrobiological Sciences DNA Bank: http://www.dna.affrc.go.jp/) using the following primers: 5' TCATGCAGCTGATCATGAGGCCCGACCCGCGGC-3' and 5' GTGCGGCCGGCAATTTCAATGGCTCTCTGTGTCGATCC-3'. The fragment was cloned into the BanHI and EcoRI sites of the pENTR2B entry vector (Invitrogen) using the In-Fusion Cloning Kit (Clontech). Unfortunately, the resulting plasmid (pENTRSSG6cDNA-defective) lacks the GC-rich region of the first half of the Os01g0179400 cDNA. Therefore, the missing region was amplified again from AK065212 using primers 5' AACCTACTGCATTGACATGGCAAGCCCGACCCGCGGC-3' and 5' TATGGCAGCCAGCCCTCCTATGAGGAGGAGCCCGCGGC-3'. The amplified fragment was inserted into the SalI and SacI sites of pENTRSSG6cDNA-defective. The SalI site is located in the vector-derived region, and the SacI site is in the middle of the cDNA. The resulting plasmid (pENTRSSG6cDNA-full) is used for the LR recombination reaction with the destination vector pLPK002 (Himmelbach et al., 2007) using the LR Cloning system (Invitrogen). The resulting plasmid was then introduced into the ss6 mutant in the cv Nipponbare background using an Agrobacterium tumefaciens-mediated method (Hiei et al., 1994).

To construct transgenic plants expressing the SS6G protein fused with GFP, SS6G cDNA was fused with the GFP gene. SS6G cDNA without a stop codon was amplified using AK065212 as a template and the following primers: 5' TCATGCAGCTGATCATGAGGCCCGACCCGCGGC-3' and 5' GTGCGGCCGGCAATTTCAATGGCTCTCTGTGTCGATCC-3'. The amplified fragment was inserted into the BanHI and EcoRI sites of the pENTR2B entry vector. Observation was carried out using a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions).

TEM Observation

The middle region of leaves and 3-DAF endosperms were fixed in 2% osmium tetroxide at 4°C for 3 h. In the case of pollens, mature anthers were fixed in the fix buffer followed by postfixation in 2% osmium tetroxide for at least 24 h at 4°C. For the maturing endosperm, to observe chloroplasts, the middle region of leaves was sampled. The procedures of resin embedding using Technovit 7100 resin (Kulzer) were described previously (Matsushima et al., 2010). Thin sections (1 m) were prepared using an ultramicrotome (EM UC7; Leica Microsystems) and diamond knives. Staining procedures were described previously (Matsushima et al., 2010). Quantification of images was performed with ImageJ 1.46r software (http://rsweb.nih.gov/ij/).

Map-Based Cloning and Microarray Analysis to Determine the ss6g Locus

To map the SS6G gene, we constructed an F2 population derived from a cross between the ss6g mutant and cv Kasalath. To select ss6g mutant seeds from the F2 population, endosperm thin sections of each F2 seed were examined by the rapid method that was developed previously (Matsushima et al., 2010). Genomic DNA of these ss6g mutant seeds was individually isolated and analyzed using simple sequence length polymorphism markers to determine the molecular markers linked to the ss6g phenotype (Temnykh et al., 2000; McCouch et al., 2002). Primers used for molecular markers are as follows: RM88, 5'-GTGCGGCCGCGAATTTTACTTGTACAGCTCGTCCATGC-3'; RM88R, 5'-AAC-ATCT-3'; RM190, 5'-TTGTGTCCTCTAAGACGAC-3'; and RM190R, 5'-TGGCAATGTTCCTGTCACTGC-3'. The amplified fragment was cloned into the BanHI and EcoRI sites of the pENTR2B entry vector. Observation was carried out using a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions).

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SS6G homologous sequences were searched through BLAST in the TAIR and RAP databases. Sequences were aligned with ClustalW (http://www.genome.jp/tools/clustalw/). Trees were constructed on conserved positions of the alignment by clustered protein sequences from plants with the neighbor-joining algorithm as implemented in MEGA 5.2 with pairwise deletion for gap filling (Tamura et al., 2011). To test inferred phylogeny, we used bootstrap replicates with 1,000 bootstrap replicates.

Analysis of Amylose Content

The polished rice grains were pulverized by Cyclotec 1093 (FOSS) to obtain powder. The powder (100 mg) was suspended in 5 ml of 50 mM NaOH and subjected to AutoAnalyzer (Technicon). In the AutoAnalyzer, the sample was mixed with 400 mM NaOH and heated at 98°C for gelatinization. The gelatinized sample was mixed with 30 mM citric acid and 85 mM ascorbic acid buffer and stained with iodine solution (3 mM potassium iodide and 0.2 mM iodine). Finally, the optical density at 620 nm was measured. A standard curve was made using potato (Solanum tuberosum) amylose standard (Sigma; A0312).

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Detection of GFP Signals in Endosperms and Pericarps of SSG6-GFP Transgenic Plants

Developing seeds at 3 DAF without husks were embedded in 5% (w/v) agarose and cross sectioned through the middle portion of the seed in 120-μm-thick sections with a vibrating blade microtome (VT-1200S; Leica Microsystems). The sections were examined using the laser scanning confocal microscope (FV1000; Olympus).

Measurement of Agronomic Traits

The third backcross generation of ssg6 (BC3F2) was grown in an experimental field at the Institute of Plant Science and Resources, Okayama University. In 2014, germinated seeds were sown on April 10, and the seedlings were transplanted into the padd field about 30 d later with a distance of 18 cm between plants and 30 cm between rows. The plants were raised according to standard practices for the time of year in Japan. Phenotypic traits were measured after harvest except for the duration to heading. The seven phenotypic traits were duration to heading, culm length, panicle length, number of panicles, number of spikelets per panicle, number of sterile spikelets per panicle, and total panicle weight.

Detection of ssg4 and ssg6 Mutations

The ssg4 mutation sites were detected by the derived cleaved-amplified polymorphic sequence primers designed as follows: 5'-ACCTAAAAGGTGTCT-TAACCTTTGAAACT-3' and 5'-AAAACCACTAACCAGCTGAGA-3'. The PCR conditions were as follows: 94°C for 2 min and 35 cycles of 94°C for 30 s, 52°C for 45 s, and 68°C for 1 min. The PCR product was digested with SpeI, and PCR products were subsequently separated by 15% PAGE and detected with ethidium bromide staining. In the case of ssg4, a PCR product (140 bp) was digested into 114 and 26 bp. In the case of the wild type, the PCR product was not digested.

To detect the ssg6 mutation, the derived cleaved-amplified polymorphic sequence primers were designed as follows: 5'-CTCATCCCCGTCGCCCTCTCT- TACTGTGC-3' and 5'-GGGTAGTAGAGCGAGTCGTCGTC-3'. The PCR conditions were as follows: 95°C for 2 min and 35 cycles of 95°C for 30 s, 57°C for 45 s, and 72°C for 1 min. The PCR product was digested with NheI, and PCR products were subsequently separated by 15% PAGE and detected with ethidium bromide staining. In the case of ssg6, a PCR product (218 bp) was digested into 189 and 29 bp. In the case of the wild type, the PCR product was not digested.

Total Protein Extraction and Immunoblotting

Two seeds at 11 DAF from wild-type cv Koshihikari and ssg6 mutants were ground in 5% volume of urea-SDS sample buffer consisting of 0.125 M Tris-HCL, pH 6.8, 8% urea, 4% (v/v) SDS, 5% (v/v) p-mercaptoethanol, and 0.02% (v/v) Bromophenol Blue supplemented with 10 μL mL-1 protease inhibitor cocktail (Sigma). Samples were extracted for 1 h at room temperature on a rotating platform and centrifuged at 20,000 g at room temperature for 10 min to remove starch. The supernatants (7.5 μL) were subjected to 7.5% SDS-PAGE. After SDS-PAGE, the proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore) by transblotter (Nihon Eido). The rest of the western-blotting procedure was performed as described by Crofts et al. (2012). Primary antibodies were used at the following dilutions: anti-SSI (Fujita et al., 2006) at 1/2,000, anti-SSIIa (Crofts et al., 2012) at 1/2,000, anti-SSIVb at 1/1,000, anti-GBSSI (Fujita et al., 2006) at 1/5,000, anti-BEI (Nakamura et al., 1992) at 1/2,000, anti-BEIIa at 1/3,000, anti-BEIIa (Nakamura et al., 1992) at 1/3,000, anti-FUL (Nakamura et al., 1996) at 1/1,000, and anti-PPH1 (Sakoh et al., 2008) at 1/10,000.

The accession number of the SSG6 full-length cDNA sequence is LC506536 in the GenBank/EMBL/DNA Data Bank of Japan databases. All other sequence data used in this article can be found in TAIR and RAP databases.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Polished grain appearance, number and size of starch granules in SGs, amylopectin chain-length distribution, and amylose content of ssg6 mutants.

Supplemental Figure S2. Semi-quantification of sugars in wild-type cv Koshihikari and the ssg6 mutant.

Supplemental Figure S3. TEM images of SGs at 3 DAF.

Supplemental Figure S4. Accumulation of starch biosynthetic enzymes in developing seeds of wild-type cv Koshihikari and ssg6 mutants.

Supplemental Figure S5. Starch morphologies in ssg6 root cap and pericarp.

Supplemental Figure S6. Number of chloroplasts per cell and number of starch granules per plastochloroplast of third and flag leaves.

Supplemental Figure S7. Chloroplast morphologies in flag leaves of ssg6.

Supplemental Figure S8. Microarray analysis of gene expression in wild-type cv Koshihikari and ssg6.

Supplemental Figure S9. SSG6 coding DNA sequence and its translated amino acid sequence in wild-type cv Koshihikari.

Supplemental Figure S10. Multiple sequence alignment of SSG6 homologs from Arabidopsis and rice.

Supplemental Figure S11. Chloroplast membrane localization of SSG6-GFP.

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We thank Makoto Kobayashi (RIKEN) and Kazuki Saito (RIKEN) for metabolite profiling analysis, Dr. Tsuyoshi Nakagawa (Shimane University) for providing Gateway vectors (pGWBA405 and pGWBI602) containing the bar gene identified by Meiji Seika Kaisha, the Leibniz Institute of Plant Genetics and Crop Plant Research for providing another Gateway vector (pIPKb002), and the Biotechnology Center of Akita Prefectural University for technical assistance.

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