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Rice mutant with enlarged starch grains

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Research area

Cell Biology
Title
Amyloplast-localized SSG4 protein influences the size of starch grains in rice endosperm

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One-sentence summary
The size of starch grains is one of the most important factors for the industrial applications of starch. A novel amyloplast-localized protein, SSG4 influences the size of starch grains in rice endosperm.
Footnotes

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ryo Matsushima (rmatsu@rib.okayama-u.ac.jp).
ABSTRACT

Starch is a biologically and commercially important polymer of glucose and is synthesized to form starch grains (SGs) inside amyloplasts. Cereal endosperm accumulates starch to levels that are more than 90% of the total weight, and most of the intracellular space is occupied by SGs. The size of SGs differs depending on the plant species and is one of the most important factors for industrial applications of starch. However, the molecular machinery that regulates the size of SGs is unknown. In this study, we report a novel rice mutant called ssg4 (substandard starch grain 4) that develops enlarged SGs in the endosperm. Enlargement of SGs in ssg4 was also observed in other starch-accumulating tissues such as pollen grains, root caps, and young pericarps. The SSG4 gene was identified by map-based cloning. SSG4 encodes a protein that contains 2,135 amino acid residues and an N-terminal amyloplast-targeted sequence. SSG4 contains a DUF490 (Domain of unknown function 490) that is conserved from bacteria to higher plants. DUF490-containing proteins with lengths greater than 2,000 amino acid residues are predominant in photosynthetic organisms such as cyanobacteria and higher plants, but are minor in proteobacteria. The results of this study suggest that SSG4 is a novel protein that influences the size of SGs. SSG4 will be a useful molecular tool for future starch breeding and biotechnology.
INTRODUCTION

Plastids are originated from the endosymbiosis of cyanobacteria and can differentiate into several forms depending on their intracellular function during the plant life cycle (Sakamoto et al., 2008). The amyloplast is a terminally differentiated plastid responsible for starch synthesis and storage. Starch forms insoluble particles in amyloplasts, referred to as starch grains (SGs). SGs are easily visualized by staining with iodine solution, and they can be observed using a light microscope. SGs are observed in storage organs such as seed endosperm, potato tubers, and pollen grains. Non-storage tissues such as endodermis and root caps also contain SGs (Morita, 2010).

Cereal endosperm accumulates high levels of starch in amyloplasts. The volume of SGs is approximately the same as the volume of amyloplasts that fill most of the intracellular space. SGs in rice (Oryza sativa) endosperm are normally 10–20 μm in diameter (Matsushima et al., 2010). One amyloplast contains a single SG that is assemblies of several dozen smaller starch granules. Each starch granule is a sharp-edged polyhedron with a typical diameter of 3–8 μm. This type of SGs is called compound SGs (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, simple SGs contain a single starch granule. 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; Matsushima et al., 2013).
The size of SGs in cereal endosperm is 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 <10 μm) coexist in the same cells (Evers, 1973; French, 1984; Jane et al., 1994; Matsushima et al., 2010). In Bromus species, intrageneric size variations of SGs are observed, in which even phylogenetic neighbors develop distinctly sized SGs (Matsushima et al., 2013). The size of SGs can be controlled by manipulating the activity of starch synthetic enzymes using transgenic plants or genetic mutants (Gutiérrez et al., 2002; Bustos et al., 2004; Ji et al., 2004; Stahl et al., 2004; Matsushima et al., 2010). However, a molecular mechanism that controls the interspecific size variations of SGs has not been resolved.

The SG occupies most of the amyloplast interior because the SG is approximately the same size as the amyloplast. The size of amyloplasts may affect the size of SGs, or vice versa. Amyloplasts and chloroplasts both develop from proplastids. The size of chloroplasts is controlled by the chloroplast binary fission division machinery, especially by the ring structures that form at the division sites (Miyagishima, 2011). Proteins involved in the ring structures have been isolated, including FtsZ, MinD, MinE, and ARC5 (DRP5B). Arabidopsis mutants that are defective in these proteins have defects in chloroplast division, and contain enlarged and dumbbell-shaped chloroplasts. In contrast to the binary fission of chloroplasts, amyloplasts divide at multiple sites and generate a beads-on-a-string structure (Yun and Kawagoe, 2009). The inhibition of chloroplast division machinery does not result in enlarged amyloplasts.
We recently developed a rapid method to prepare thin sections of endosperm (Matsushima et al., 2010). Using this method, SGs in mature endosperm can be easily and clearly observed. We performed genetic screening for rice mutants defective in SG morphologies and sizes. One of the isolated mutants, *ssg4* (*substandard starch grain 4*), develops enlarged SGs in its endosperm. In this study, we characterized *ssg4* phenotypes and identified the responsible gene. *SSG4* encodes a protein containing 2,135 amino acid residues and an N-terminal plastid-targeted sequence. The DUF490 (Domain of unknown function 490) was found at the C-terminus of SSG4, where the *ssg4* mutation was located. This suggests that SSG4 is a novel factor that influences the size of SGs, and has potential as a molecular tool for starch breeding and biotechnology.

**RESULTS**

**Enlarged SGs in *ssg4* mutant endosperm**

The chalkiness of seeds was a distinguishing phenotype of *ssg4* grains when compared to wild-type grains of Nipponbare (Fig. 1, A–D). Seed size was slightly smaller in *ssg4* than in Nipponbare, especially with respect to seed width and depth (Fig. 1E). The iodine-stained thin sections of mature endosperm clearly showed enlarged SGs in *ssg4* endosperms (Fig. 1, F–I). Quantification of the areas occupied by SGs in the thin sections showed that SGs were approximately 6-fold larger in *ssg4* than in Nipponbare (Fig. 1J).
The endosperm is a triploid tissue generated by the fusion of sperm and the binucleate central cell of the female gametophyte (Li and Berger, 2012). Therefore, endosperm has four possible genotypes at one gene locus, namely, AAA, AAa, Aaa, and aaa. We performed reciprocal crosses to obtain two distinct heterozygous seeds of $SSG4SSG4ssg4$ and $SSG4ssg4ssg4$. Chalkiness was not observed in the endosperm of either heterozygote (Supplemental Fig. S1). The SG sizes of $SSG4SSG4ssg4$ and Nipponbare seeds did not significantly differ, whereas the SG sizes of the $SSG4ssg4ssg4$ seeds were slightly larger than those of Nipponbare (Fig. 1J). This indicated that two wild-type alleles supplied from the female gametophyte were sufficient for the formation of normal-sized SGs, whereas one copy of the $SSG4$ allele supplied by the sperm was functional but not sufficient for the formation of normal-sized SGs. We next examined starch accumulation in $ssg4$ grains. The total amount of starch was lower in $ssg4$ seeds than in wild-type seeds (Fig. 1K). No significant difference in the gelatinization properties of $ssg4$ starch compared to wild-type starch was observed; therefore, the structural properties of starch were similar in $ssg4$ and Nipponbare (Table I). This result was consistent with previous work showing that the amylopectin chain-length distribution of $ssg4$ starch is normal (Matsushima et al., 2010).

Arabidopsis phosphoglucomutase ($pgm$) mutant contains small amounts of starch in leaves, while exhibits high levels of accumulation of soluble sugars, such as sucrose, D-glucose, and D-fructose (Blasing et al., 2005). This is explained by the defective conversion of photosynthate into starch in $pgm-1$ leaves. Less starch accumulation in $ssg4$ seeds also might cause the abnormal level of sugar accumulation.
We analyzed the soluble sugars in *ssg4* and Nipponbare seeds by using gas chromatography-time-of-flight/mass spectrometry (GC-MS). Levels of sucrose and D-glucose were much higher in *ssg4* seeds than those in Nipponbare seeds (Supplemental Fig. S2, A and B). While, the D-fructose level was less abundant in *ssg4* seeds than that in Nipponbare seeds (Supplemental Fig. S2C).

Rice grains require more than 1 month for full ripening after flowering. During this period, a large number of SGs are developed and fill the endosperm. To investigate when the enlarged SGs were developed in *ssg4* mutant, we focused on early developing seeds at 3, 5, and 7 days after flowering (DAF). Seed enlargement from 3–7 DAF in Nipponbare and *ssg4* was essentially the same (Fig. 2, A–F). By contrast, the sizes and numbers of SGs from 3–7 DAF in Nipponbare and *ssg4* were different (Fig. 2, G–L). At 3 DAF, most SGs in the *ssg4* endosperm were larger than those in Nipponbare (Fig. 2, G and J) and occupied an area that was more than 3-fold larger than that occupied by SGs in Nipponbare (Fig. 2M). At 7 DAF, the area occupied by SGs was more than 5-fold larger in *ssg4* than in Nipponbare. When the SGs were assumed to be spherical, the volume of SGs at 7 DAF was approximately 10-fold larger in *ssg4* than in Nipponbare. The number of SGs showed the opposite pattern to the sizes of SGs (Fig. 2N) and was lower in *ssg4* than in Nipponbare at all days tested. At 3–7 DAF, the number of SGs in *ssg4* was one-third less than the number in Nipponbare (Fig. 2N).

We also investigated *ssg4* endosperms at 5 DAF by transmission electron microscope (TEM) (Supplemental Fig. S3). Morphologies of *ssg4* SGs in TEM images were spherical like the iodine-stained SGs in Figure 2.
SG morphologies in other tissues

Endosperm tissue accumulates the highest levels of starch in rice plants. Other tissues also accumulate SGs, including pollen grains, root caps, and pericarps. We examined SG morphologies in these tissues in ssg4 mutants. Pollen grains were immersed in iodine solution to stain SGs, and many rod-like SGs were visualized in Nipponbare pollen grains (Fig. 3A). By contrast, ssg4 SGs in pollen were more spherically shaped (Fig. 3B). In both cases, pollen SGs displayed different morphologies from those of endosperm SGs. When pollen grains were squashed under cover slips, SGs were released and the morphologies were clearer (Fig. 3, C and D). Scanning electron micrographs of released SGs also showed that the SG morphologies were different in Nipponbare and ssg4 (Fig. 3, C and D, insets). Most SGs in pollen grains of both Nipponbare and ssg4 appear to be simple SGs. SGs were slightly larger in ssg4 pollen grains than in Nipponbare pollen grains (Fig. 3E). Root caps developed many SGs that were the compound type (Fig. 3, F–I). The pericarp is the wall of the mature ovary and it surrounds the entire seed. In early developing rice seeds, many compound SGs developed in the pericarp (Fig. 3, K–N). The SGs in the ssg4 pericarp were more spherical than those in the Nipponbare pericarp (Fig. 3, K–N). In root caps and pericarps, ssg4 SGs were more than 2-fold larger than Nipponbare SGs (Fig. 3, J and O). All these results suggest that the ssg4 mutation affects the size of SGs in pollen grains, root caps, pericarp, and endosperm.

The third leaves of ssg4 mutants showed a variegated phenotype (Fig. 4, A and
B. We speculated that chloroplasts might also be affected by the $ssg4$ mutation. To visualize chloroplasts, thin sections of third leaves from young seedlings were double-stained with methylene blue and basic fuchsin (Fig. 4, C–F). Nipponbare chloroplasts displayed elongated, lens-like shapes, whereas those of $ssg4$ were more spherical (Fig. 4, E and F). The areas of chloroplasts were approximately 2-fold larger in $ssg4$ than in Nipponbare (Fig. 4G). These results indicate that the $ssg4$ mutation affects the size of chloroplasts and amyloplasts. We also investigated $ssg4$ chloroplasts by TEM to observe the chloroplastic ultrastructures, such as starch granules, grana stacks and envelope membranes. The size of starch granules in $ssg4$ chloroplasts showed similar sizes with those in the Nipponbare chloroplasts (Fig. 4, H and I). Grana stacks and envelope membranes were not affected in $ssg4$ chloroplasts (Fig. 4, J and K). In contrast to the third leaves, $ssg4$ flag leaves didn’t show variegated phenotype (Supplemental Fig. S4, A and B). Shapes of chloroplasts in the flag leaves didn’t show much difference between Nipponbare and $ssg4$ (Supplemental Fig. S4, C-F). Areas of chloroplasts in the $ssg4$ flag leaves were a little larger than those in the Nipponbare, but not to the degree in the third leaves (Supplemental Fig. S4G). TEM showed that the chloroplastic ultrastructures were not affected in the $ssg4$ flag leaves (Supplemental Fig. S4, H-K).

**Genetic analysis and map-based cloning of the SSG4 gene**

When $ssg4$ was crossed with Nipponbare, approximately half of the pollen grains of the F1 plants had $ssg4$ phenotypes (Table II). This indicates that $ssg4$ behaves
in a gametophytic manner in pollen grains. The \textit{ssg4} phenotype in endosperm tissue was completely penetrant in \textit{ssg4} selfed progeny and segregated as a single recessive allele in F2 progenies (Table III). We identified the \textit{SSG4} gene using conventional map-based cloning. We mapped the \textit{ssg4} mutation within a 62 kb region on chromosome 1 (Fig. 5A). Ten open reading frames are expected in this region according to the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). We identified a base change in \textit{Os1g0179400} gene of \textit{ssg4} mutant. \textit{ssg4} mutant carries guanine (G) to \textit{adenine (A)} transition at the position of 4,139,234 (Os-Nipponbare-Reference-IRGSP-1.0-based position) on chromosome 1. The G to A transition is consistent with an EMS-induced mutation. A previously isolated cDNA clone of \textit{Os1g0179400} (AK063507) encodes a protein containing 1,022 amino acid residues with a DUF490, according to the Pfam database (Punta et al., 2012). SSG4 is similar to the \textit{emb2410} (embryo defective 2410) protein in Arabidopsis. Although AK063507 was registered as a full-length cDNA, all other homologous proteins from Arabidopsis, \textit{Brachypodium}, and maize contain more than 1,000 additional amino acids at the N-termini compared to the \textit{Os1g0179400} protein predicted from AK063507. This raises the possibility that the reported 5' terminus of AK063507 is incorrect, and that a longer protein is encoded by the real \textit{Os1g0179400} full-length cDNA. To investigate this possibility, we performed a 5' RACE experiment to determine the 5' end of \textit{Os1g0179400}. The RACE experiment showed that the 5' end of \textit{Os1g0179400} is far longer than that of AK063507. The new full-length cDNA of \textit{Os1g0179400} is derived from 23 exons and the 5' UTR at the 5' terminus (Fig. 5B). The deduced protein had 2,135 amino acid residues and contains a
putative plastid-targeting sequence at the N-terminus. For the complementation test, we cloned the genomic sequence of 14,263 nucleotide base pairs starting from the putative first ATG to 1,299 nucleotides downstream of the stop codon of the Os01g0179400 gene. We could not clone the promoter sequence of SSG4 because it was unstable and caused deletions during plasmid construction. Therefore, we used the maize ubiquitin 1 promoter to express the Os01g0179400 genomic clone (Himmelbach et al., 2007). The genomic clone was introduced into the ssg4 mutant, and the transgenic plants that were homozygous for the transgene were isolated and named UBi:SSG4genomic/ssg4. The sizes and morphologies of SGs in transgenic UBi:SSG4genomic/ssg4 endosperm and pollen grains were very similar to those in Nipponbare (Fig. 6). This indicates that the SG phenotypes in endosperm and pollen grains were completely rescued by the transgene. We conclude that Os1g0179400 is the gene responsible for the ssg4 mutation.

SSG4 had a putative plastid-targeting sequence in its N-terminal region (Fig. 5B). Other than the plastid-targeting sequence and DUF490, no other functional domains were identified in SSG4 protein. Phylogenetic analysis showed that DUF490 domains from photosynthetic organisms form a different group separate to proteobacterial DUF490 domains (Fig. 5C). The ssg4 mutation substitutes the glycine residue at position 1,924, which is located within the DUF490, with a serine residue. This glycine residue is conserved from proteobacteria to higher plants, which suggests it is important for the function of the DUF490 (Fig. 5D).

Expression patterns of the SSG4 gene
The expression patterns of SSG4 in various tissues of different developmental stages were investigated using real-time quantitative PCR by three different sets of primers (Supplemental Fig. S5A). P1, P2 and P3 primer sets were used to detect the first, middle and the last exons of SSG4 gene, respectively (Fig. 5B). All tissues except for third leaves were sampled from plants grown in paddy field. To obtain third leaves, plants were grown in greenhouse. Real-time quantitative PCR showed that SSG4 was expressed in all tissues examined in both Nipponbare and ssg4. This suggests that SSG4 is needed at all developmental stages. During early seed development, SSG4 transcripts started to accumulate at 4 DAF in Nipponbare but the accumulation was delayed in ssg4. At 5–7 DAF, the expression of SSG4 continued to increase in both Nipponbare and ssg4, reaching a high level. In young plants, the third leaves in Nipponbare had a high level of SSG4 expression, which was approximately twice as high in ssg4. The much expression of SSG4 gene in third leaves compared to the flag leaves in Nipponbare may reflect the more requirements of SSG4 gene in third leaves. This is consistent with the severe enlargement of chloroplasts in the third leaves compared to the flag leaves in ssg4 (Fig. 4; Supplemental Fig. S4). The expression patterns obtained using the P2 and P3 primer sets were approximately the same as that obtained using the P1 primers (Supplemental Fig. S5, B and C). This indicates that all three primer sets amplified the same cDNA species. Therefore, the long full-length SSG4 cDNA determined in this study should be the dominant cDNA species.

**Subcellular localization of SSG4 protein**
The target prediction programs Target P (Emanuelsson et al., 2007) and WoLF PSORT (Nakai and Horton, 2007) predicted that the SSG4 protein is targeted to chloroplasts and has a putative transit peptide at the N-terminus. To confirm the chloroplast localization of SSG4, we attempted to construct the SSG4 gene fused with GFP. We used the N-terminal coding region (639 bp) of the SSG4 cDNA instead of the full-length cDNA because the full-length cDNA sequence strongly inhibited bacterial growth and was difficult for plasmid construction. The plasmid construct containing the N-terminus of SSG4 fused to GFP was designated SSG4N-GFP. When SSG4N-GFP transiently expressed in Nicotiana benthamiana leaves, the SSG4N-GFP signals were detected inside chloroplasts and the patterns were very similar to the stroma-localized GFP (Supplemental Fig. S6). This result indicates that SSG4N-GFP was mainly localized in stroma of chloroplasts.

We constructed stable transgenic rice plants expressing SSG4N-GFP gene under the control of the maize ubiquitin 1 promoter. In SSG4N-GFP plants, SSG4N-GFP fluorescence was detected in pollen grains, endosperm, and pericarp (Fig. 7). In pollen grains, SSG4N-GFP fluorescence was observed as a ring-like structure (Fig. 7, A–F). Differential interference contrast images of pollen showed that the ring-like GFP fluorescence surrounded rod-shaped structures (Fig. 7, E and F), which are likely to be SGs as their morphologies are consistent with the iodine-stained SGs shown in Figure 3. In developing endosperm and pericarp, SSG4N-GFP co-localized with the amyloplasts whose interiors contained compound SGs (Fig. 7, G–I). SSG4N-GFP was excluded from the SGs and accumulated in non-starch areas (Fig. 7, J–L). In endosperm
SGs, each starch granule is compactly assembled, which might prevent the SSG4N-GFP protein from entering the intergranule space. SSG4N-GFP accumulated in the spaces between SGs and amyloplast membranes (Fig. 7, J–L, arrowheads). This space will correspond to the stroma in endosperm amyloplasts. By contrast, SSG4N-GFP fluorescence accumulated in the space between the starch granules in pericarp SGs (Fig. 7, M–O). This suggests that starch granules in pericarp SGs are loosely assembled, which allows SSG4N-GFP to enter the intergranule space. Taken together, these data show that SSG4N-GFP is localized in the amyloplasts of various tissues, and suggest that SSG4 is an amyloplast-localized protein with an N-terminal plastid-targeting signal.

**Accumulation of proteins involved in chloroplast division in ssg4 seeds**

In rice, the *arc5* mutant is the only mutant reported to be defective in chloroplast division. However, the *arc5* endosperm does not produce spherical amyloplast with increased diameter such as *ssg4* (Yun and Kawagoe, 2009). The proteins involved in chloroplast division (FtsZ1, FtsZ2, MinD, and MinE) accumulated at the same level in *ssg4* and Nipponbare (Supplemental Fig. S7). Therefore, we speculate that SSG4 is not directly involved in the regulation of plastid division.

**Protein length diversity of DUF490-containing proteins**

In the InterPro protein sequence analysis and classification database (Hunter et al., 2012), 4,546 DUF490-containing proteins are registered. Proteins containing
DUF490 are found from bacteria to higher plants, but not in animals. TamB (translocation and assembly module B) is well-characterized DUF490-containing protein in proteobacteria and is responsible for the insertion and assembly of outer membrane proteins (Selkriig et al., 2012). Out of the 4,546 DUF490-containing proteins, proteobacterial proteins predominate and include 3,566 proteins, whereas 166 proteins are registered for cyanobacteria and 41 proteins are registered for Viridiplantae (green algae and land plants). A comparison of the lengths of these DUF490-containing proteins showed that proteins from cyanobacteria and Viridiplantae are clearly longer than those from proteobacteria (Supplemental Fig. S8, A–C). The lengths of most proteobacterial proteins are approximately 1,300 amino acid residues. For examples, TamBs from *Citrobacter rodentium*, *Salmonella enterica* and *E. coli* are all 1,259 amino acid residues (Selkriig et al., 2012). While, majority of cyanobacterial and Viridiplantae proteins are around 2,000 amino acid residues. The difference of protein length distributions among proteobacteria, cyanobacteria and Viridiplantae were statistically significant (Steel-Dwass analysis; proteobacteria and cyanobacteria, *P* < 0.001; proteobacteria and Viridiplantae, *P* = 0.005; cyanobacteria and Viridiplantae, *P* = 0.542). Several DUF490-containing proteins of Viridiplantae with around 2,000 amino acid residues are predicted to target plastids. Therefore, the longer DUF490-containing proteins may be needed for photosynthetic organisms and organelles.

**DISCUSSION**
Regulation of SG sizes by SSG4

The size of SG is one of the most important characteristics of starch for industrial applications (Lindeboom et al., 2004). Small starch granules are used to replace fat in food applications because aqueous dispersions of small starch granules exhibit fat-mimetic properties (Malinski et al., 2003). In maize and cassava crops, larger starch granules are desirable because they improve the final yield after wet-milling purification (Gutiérrez et al., 2002).

In this study, we characterized ssg4 phenotypes that show enlarged SGs in endosperm, pollen grains, root caps, and pericarps (Fig. 1, 2 and 3). SSG4 was identified as the gene that influences the SG sizes (Fig. 5). An amino acid substitution from glycine to serine in DUF490 of SSG4 increased the size of SGs. However, enlargement of SGs did not result in the direct expansion of starch granules in ssg4, because SGs in ssg4 endosperm are the compound SG type. The information obtained in this study will be applicable to other crops for the production of larger starch granules. For simple SGs, the size of SG is consistent with the size of starch granules. Therefore, the enlargement of SGs will directly generate larger starch granules. Barley, maize, and sorghum develop simple SGs, and all these species have homologs of SSG4. These homologs have a conserved glycine residue at the mutation site of ssg4 reported in this study, same as the wild-type rice cultivar Nipponbare. Therefore, introduction of this same mutation into these crops or down-regulation of homologs of SSG4 will produce larger starch granule.

A number of mutants defective in starch biosynthetic enzymes of endosperms
have been isolated in several plant species (Walker and Merritt, 1969; Jarvi and Eslick, 1975; Satoh and Omura, 1981; Yano et al., 1984; Satoh et al., 2003; Satoh et al., 2003; Kang et al., 2005; Fujita et al., 2007; Satoh et al., 2008; Fujita et al., 2009, Dvonch, 1951 #62). Some of these mutants exhibit distinct SG morphologies in endosperms compared to those of the wild-type plants. Mutations in amyllopectin branching enzyme IIb reduce the size of SGs in rice and maize endosperm (Yano et al., 1985; Li et al., 2007; Matsushima et al., 2010). While, Arabidopsis mutant of starch synthase IV forms one huge starch granule per chloroplast in leaves (Roldan et al., 2007). Starch synthase IV is suggested to be involved in the process of initiation of the starch granule and in the priming of starch synthesis (Szydlowski et al., 2009; D'Hulst and Merida, 2010). However, the role of starch synthase IV in cereal endosperms has remained unknown so far.

**Subcellular localization of SSG4**

The SSG4 N-terminal sequence targeted GFP to the stroma of chloroplasts and amyloplast in various tissues (Supplemental Fig. S6 and Fig. 7). While, proteomic analysis of cyanobacteria showed that SSG4 cyanobacterial homologs are localized in the outer membrane (Moslavac et al., 2005). The Prediction of Transmembrane Regions and Orientation (TMpred) program (http://www.ch.embnet.org/software/TMPRED_form.html) predicted three transmembrane regions in SSG4 sequence (amino acids 104–127, 517–540, and 1463–1485). The first region had the highest confidence interval. However, the first
region should not be a transmembrane domain because it is included in the SSG4N-GFP. The latter two regions may be important for the intraplastidic localization of SSG4 and may target SSG4 to membranes.

**Possible functions of SSG4 protein**

To date, *SSG4* homologs of photosynthetic organisms have not been functionally characterized. T-DNA knockout mutations of the Arabidopsis homolog of *SSG4* (*At2g25660*), denoted as *emb2410*, arrest embryo development at globular stage (Meinke et al., 2008). Many embryo-defective mutants with the disruption of plastid-targeted proteins have been shown to exhibit the impaired plastid development (Hsu et al., 2010; Bryant et al., 2011). In the case of Arabidopsis *arc1* mutant, the weak allele mutant has smaller, more numerous chloroplasts than the wild type, while the strong T-DNA insertion allele causes embryo-lethality (Kadirjan-Kalbach et al., 2012). ARC 1 likely functions in an essential process of plastid development that may be coupled with plastid division. In a similar way, the essential function of *At2g25660* gene for embryogenesis raises the possibility that function of SSG4 is more involved in plastid development rather than the direct role for SG size control. The pleiotropic effect of *ssg4* mutation on chloroplast organization in third leaves and conservation of DUF490-containing proteins in cyanobacteria that does not develop SGs supports this idea (Fig. 4 and 5).

Septum-like structures have been suggested to exist between starch granules during the formation of compound SGs (Yun and Kawagoe, 2010). The successive
synthesis of septa during plastid division promotes the formation of compound SGs. SGs in endosperms, root caps and pericarps were compound type, while SGs in pollen grain were simple type (Fig. 1, 2 and 3). The enlargement of SGs by ssg4 mutation was extreme in compound SGs compared to the simple SGs (Fig. 3). Therefore, SSG4 may be related to the septum formation and have some specific roles in the compound nature of dividing plastids. The amino acid substitution in ssg4 is the first non-lethal mutation in DUF490-containing proteins of photosynthetic organisms. Future studies of SSG4 will reveal more detailed information about the DUF490 function in higher plants.

MATERIALS AND METHODS

Plant materials and growth condition

*Oryza sativa* spp *japonica* cv Nipponbare and the indica variety Kasalath were used as wild-type plants. *ssg4* mutant was previously isolated from EMS-treated Nipponbare M2 population (Matsushima et al., 2010). We backcrossed the *ssg4* mutant with Nipponbare and their progenies with *ssg4* phenotypes were used in this study. Rice plants were grown at an experimental paddy field of Institute of Plant Science and Resources, Okayama University under natural condition or at 28°C in a greenhouse.

Characterization of grain appearance, sizes and starch amount

Matured dry seeds and maturing young seeds were photographed with a macro-microscope (MVX10, Olympus, Tokyo, Japan) and a digital camera (DP72,
Olympus). The sizes of grains were measured by vernier. The total amount of starch was measured by enzymatic methods using a Total Starch Assay Kit (Megazyme International Ireland, Wicklow, Ireland).

**Thermal properties of starch**

Dried rice grain was de-hulled, crushed with pliers and hand-homogenized using motor and pestle. The weighed starch (3 mg) was placed in a silver sample cup (560–003, Seiko Instruments, Tokyo, Japan), mixed with 9 μl of distilled water, and sealed. Gelatinization properties of the starch were analyzed by a differential scanning calorimeter (DSC-6100, Seiko Instruments, Tokyo, Japan). The heating rate was 3°C min\(^{-1}\) over a temperature range of 5 – 90°C.

**Semi-quantification of D-glucose, D-fructose and sucrose levels**

Extracts from the matured seeds of Nipponbare and the ssg4 mutant (equivalent of 5 mg) were subjected to GC-MS as described previously (Kusano et al., 2007). Peaks of D-glucose, D-fructose and sucrose in each analyte were identified by comparing retention indecies and the mass spectra of the corresponding authentic standards.

**Thin sections of Technovit 7100 resin of endosperm and staining**

For matured dry seeds, approximately 1 mm cubic blocks were cut from the center region of the endosperm and fixed in FAA solution containing 5% (v/v) formalin, 5% (v/v) acetic acid 50% (v/v) ethanol, for at least 12 h at room temperature. For
maturing endosperm, approximately 1-mm blocks were cut out from the maturing endosperm at 3, 5 and 7 days after flowering and fixed in 3% (v/v) glutaraldehyde in 20 mM cacodylate buffer (pH 7.4) for at least 24 hr at 4°C. To observe root caps, the seminal root tips (1 mm) were cut out and fixed in the same buffer as for the maturing endosperm. To observe chloroplasts, middle region of leaves were sampled. After fixations, samples were subsequently dehydrated and then embedded in Technovit 7100 resin (Kulzer and Company, Wehrheim, Germany) as described previously (Matsushima et al., 2010). The embedded samples were cut in 1 μm sections with an ultramicrotome (LEICA EM UC7, Leica Microsystems, Tokyo, Japan) and diamond knives, and then dried on coverslips. To stain SGs, thin sections were stained with 40-times diluted Lugol solution (iodine/potassium iodine solution, MP Biomedicals, Eschwege, Germany) in deionized water for at least 5 seconds and subsequently examined under a microscope (AX70, Olympus). Quantifications of amyloplast areas were analyzed with the NIH Image J 1.46r software (http://rsbweb.nih.gov/ij/). To stain the chloroplast in leaves, the Technovit sections were double stained with 0.026% (w/v) methylene blue and basic fuchsin.

Transmission electron microscopic observation

Middle region of third leaves of seedling and 5-DAF endosperms were fixed in 2% paraformaldehyde (w/v), 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) at 4°C overnight followed by post fixation with 2% osmium tetroxide at 4°C for 3 h. Samples were subsequently dehydrated using a graded ethanol series and
infiltrated with propylene oxide. The samples were then embedded in Quetol-651 resin (Nisshin EM Co., Tokyo, Japan). The embedded samples were ultra-thin sectioned at 70 nm with a diamond knife and sections were placed on copper grids. They were stained with 2% (w/v) uranyl acetate for 15 min, and then they were secondary-stained with lead stain solution for 3 min. The grids were observed by a transmission electron microscope (JEM-1400Plus, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (VELETA, Olympus Soft Imaging Solutions GmbH).

**Observation of pollen grains**

To obtain the iodine-stained matured pollen grains, anthers just before anthesis were disrupted with forceps in the diluted Lugol solution on a glass slide, the released pollen grains were subsequently examined under the microscope (AX70). Furthermore, pollen grains were squashed by putting gentle pressure on a coverslip to release SGs from pollen vegetative cells. The released SGs were also observed with a scanning electron microscope (SEM, Quanta 250, FEI, Oregon, USA).

**Map-based cloning of SSG4 gene**

For mapping the SSG4 gene, we constructed an F2 population derived from a cross between ssg4 mutant and Kasalath. To select ssg4 mutant seeds from the F2 populations, endosperm thin sections of each F2 seed were examined by the rapid method that was developed previously (Matsushima et al., 2010). The genomic DNA of
these $ssg4$ mutants was individually isolated and analyzed using simple sequence length polymorphism markers to determine the molecular markers linked to $ssg4$ phenotype (Temnykh et al., 2000; McCouch et al., 2002). Primers used for molecular markers are as follows. Marker1169: 5’-TAAGACTGAACCTAATGATTGTGT-3’ and 5’-AAAAACATATAATCCAAAACGTTAG-3’, Marker13025: 5’-TAGGAGAGGAGAGAAGTTTGTG-3’ and 5’-GTTTAGGATCGCTACCAAATAG-3’.

The $SSG4$ locus was mapped in the 62-kb region between Marker1169 and Maker13025 on the short arm of chromosome 1. We determined the nucleotide sequence of several candidate genes in this region, and identified a single base substitution in a candidate $Os01g0179400$ gene.

**Five-prime RACE experiment**

The multiple alignments between Os01g0179400 and other plant homologous proteins indicated that the cDNA clone (AK063507) for $Os01g0179400$ gene should be missing the 5’ end of the predicted ORF. Therefore, the missing part was obtained using 5’ RACE experiment using the SMARTer RACE cDNA amplification kit (Clontech Laboratories, CA, USA), according to the manufacturer’s instructions. The first-strand cDNA were synthesized from total RNA of DAF-5 developing seeds or 7-day-old young seedlings of Nipponbare. The following primers used as gene specific primers; 5’-TGACAACAGCCTCCTCGGTATGG-3’. The sequence of the obtained PCR fragment was used as templates for direct sequencing using a Big Dye Terminator v3.1
Cycle Sequencing Kit (Applied Biosystems, CA, USA) and a genetic analyzer (Applied Biosystems 3130xl).

**Plasmid construction**

For the complementation of ssg4 mutant, a genomic fragment containing *Os01g0179400* gene was cloned into the pIPKb002, a binary vector for transformation of cereals (Himmelbach et al., 2007). The genomic fragment is 14,263-nucleotide base pairs starting from the putative first ATG and up to the 1299 nucleotide downstream of the stop codon of *Os01g0179400* gene (Chr. 1; 4,127,368 - 4,141,631). The genomic fragment was amplified separately as two fragments to construct the plasmid. First, 3’ half of the genomic region (4,131,703 - 4,141,631) was amplified using the following primers; 5’-TCAGTCGACTGGATCCAATGGGCGGTGGTTTATCTCAAAA-3’ and 5’-GTGCGGCCGCGAATTGGGAAATGGAAAGAACCTAGATTGG-3’. The fragment was cloned into the BamHI and EcoRI sites of pENTR2B entry vector (Invitrogen, CA, USA) using In-Fusion Cloning Kit (Clontech). The resulting plasmid is called pENTR-latter. The remaining 5’ half of the genomic region (4,131,703 - 4,133,132) was amplified using the following primers; 5’-AACCAATTCAGTCGAATGTCCCACTGCCTCCGGGCGTCGC-3’ and 5’-TAGTCTCTCCATTGAGCTGCTCTGATCCGTTTT-3’ (*Sac* I site is underlined). The amplified fragment was inserted into the SalI and SacI sites of pENTR-latter. The SalI site is located in the vector-derived region and SacI site is in the middle of the genomic region. The resulting plasmid, pENTR-full is used for the LR recombination reaction.
with the destination vector pIPKb002 using the Gateway system (Invitrogen). The resulting plasmid was then introduced into the ss g4 mutant using an Agrobacterium tumefaciens–mediated method (Hiei et al., 1994). To construct the transgenic plants expressing SSG4 protein fused with GFP, cDNA encoding the SSG4 N-terminal fragment (1-213 a.a.) was amplified and cloned into the pENTR2B entry vector (Invitrogen) together with GFP gene. SSG4 N-terminal coding region (SSG4N, 639 bp) was amplified by PCR amplification using the full length cDNA as a template and the following primers; 5’-AACCAATTCAATCGAATGTCCCTGCCTCCGGGCGTCGC-3’ and 5’-GCCCTTGCTCACCATCTCGGACAGCACGGCGTCGACGACG-3’. GFP gene was amplified from LAT52-GFPN plasmids (Matsushima et al., 2008) using the following primers; 5’-ATGGTGAGCAAGGGCGAGGAGCT-3’ and 5’-AAGCTGGGTCTAGATTTACTTGTACAGCTCGTCCATGC-3’. Both fragments were connected and inserted into the Sal I and EcoR V sites of pENTR2B vector using the In-Fusion Cloning Kit. In the resulting plasmid, SSG4N was connected to the GFP gene (SSG4N-GFP). The resulting plasmid (pENTRSGG4N-GFP) was used for the LR reaction with the destination vector pIPKb002. The resulting plasmid (pK02SSG4NTM-GFP) was then introduced into Nipponbare. pENTRSGG4N-GFP was also used for the LR reaction with the destination vector pGWB2 (Nakagawa et al., 2007) to introduce SSG4N-GFP gene under the control of CaMV 35S promoter. The resulting plasmid (pG2SSG4N-GFP) was used for the agrobacterium-mediated transient transformation of Nicotiana benthamiana.
**Phylogenetic analysis**

Sequences containing DUF490 domain were searched through BLAST in the GenBank/EMBL/DDBJ databases and the MIPS database (http://www.helmholtz-muenchen.de/en/ibis). Sequences were aligned with ClustalW (http://www.genome.jp/tools/clustalw/), followed by manual alignment. 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 bootstraps with 1000 bootstrap replicates.

**Expression pattern of SSG4 gene**

Different tissues, including developing seeds, anthers, pistils, young panicles, third and flag leaf blades were sampled. Except for third leaf blades, all tissues were obtained from the plants grown in the paddy field from mid May to the end of September. To obtain the third leaf blades, plants were grown in greenhouse. For developing seeds, maturing seeds were sampled at 1, 2, 3, 4, 5 and 7-day after flowering. The anthers and pistils were sampled from spikelets on the primary rachis-branches when the distance between auricles of the last two leaves was 12 cm. Young panicles were sampled when at their length were 3.5 - 4.5 cm. All samples were frozen in liquid nitrogen for RNA extraction. Extraction of total RNA was done using an RNeasy plant mini kit (Qiagen). One hundred sixty nanogram of total RNA was used for first-strand cDNA synthesis using a ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (TOYOBIO,
Osaka, Japan). The expression was determined with THUNDERBIRD SYBR qPCR Mix kit (TOYOBO) on Light Cycler 2.0 (Roche Diagnostics).

The PCR conditions were as follows; 95°C for 30 sec, and 40 cycles of 95°C for 5 sec and 55°C for 30 sec. LightCycler Software (Ver. 4.0, Roche Diagnostics) was used to quantify PCR reactions. HistoneH3 was used as an internal control (Sasaki et al., 2011).

Data shown are the average and standard deviations of three biological replicates. The primers used as follows:

P1, 5’-CATATTTTCAAAACGAGTGTAGTG-3’ and 5’-AATCCATAGATGTTGTCAGAGT-3’;
P2, 5’-TGCTGATTTATATGGCATTAGAG-3’ and 5’-GACAAATTAATATCCATAGCAGAAT-3’;
P3, 5’-ACAATATATCTTTTGCTACTGAGGT-3’ and 5’-GTTAGCTGATATATGAGTGACCA-3’;
HistoneH3 primer, 5’-GGTCAACTTGTTGATTCCCCTCT-3’ and 5’-AACCGCAAAATCCAAAGAACG-3’.

**Agrobacterium-mediated transient transformation of Nicotiana benthamiana**

Suspensions of transformed GV3101 bacteria were adjusted to an OD$_{600}$ of 0.6 in MES buffer (10 mM MgCl$_2$, 10 mM MES, pH 5.6), and acetylsyringone was added to a final concentration of 20 μM. Bacterial suspensions were then maintained at room temperature for 2–3 hr. Infiltrations were conducted by gently pressing a 1-ml disposable syringe to the abaxial surface of fully expanded leaves that were approximately 3.5 cm
wide and slowly depressing the plunger. A sufficient amount of bacterial suspension was used to completely infiltrate the leaves and give a water-soaked appearance. Following the infiltration, plants were maintained in a growth chamber at 25°C with a 12 hr /12 hr light/dark photoperiod. Leaves were examined by microscopy between 50 hr and 90 hr post-infiltration. For chloroplast stroma-localized GFP, plasmid (pL12-GFP) expressing GFP fused with transit peptide of chloroplast ribosomal protein L12 was used (Arimura et al., 1999). For chloroplast envelope-localized GFP, plasmid (pCor413im1-GFP) expressing GFP fused with Cor413 chloroplast inner envelope membrane protein 1 was used (Okawa et al., 2008). GFP signals were detected using a laser scanning confocal microscope (FV1000, Olympus).

Detection of GFP signals in endosperms and pericarps of SSG4N-GFP transgenic plants

Developing seeds (3-DAF) without husks were embedded in 5% (w/v) agarose and cross-sectioned through the middle portion of the seed in 150 μm-thick sections with a Vibrating Blade Microtomy (VT-1200S, Leica Microsystems, Tokyo, Japan). The sections were incubated in phosphate-buffered saline, and the samples were examined using the laser scanning confocal microscope (FV1000).

Total protein extraction and immunoblotting
For protein extraction from developing seeds, samples were homogenized in the extraction buffer (10 μl mg\(^{-1}\)) consisting of 50 mM Tris–HCl (pH 6.8), 8 M urea, 4% (w/v) SDS, 20% (v/v) glycerol and 5% (v/v) β-mercaptoethanol (35 μl mg\(^{-1}\)) using a plastic homogenizer. After centrifugation at 12,000 \(\times g\) for 5 min, proteins were separated by SDS–PAGE in pre-cast 10–20% polyacrylamide gel (ATTO, Japan). The gels were blotted onto a polyvinylidene fluoride (PVDF) membrane for immunoblotting with antibodies and ECL (GE Healthcare). Antibodies were prepared previously (Yun and Kawagoe, 2009).

**Accession Numbers**

The accession number of the SSG4 coding DNA sequence is AB856288 in GenBank/EMBL/DDBJ databases. All other sequence data used in this article can be also found in GenBank/EMBL/DDBJ databases and the UniProt Knowledgebase (UniProtKB).

**Supplemental Data**

The following material is available in the online version of this article.

**Supplemental Figure S1.** Grain chalkiness of \(ssg4\) and heterozygous mutant seeds.

**Supplemental Figure S2.** Semi-quantification of sucrose, D-glucose and D-fructose levels in Nipponbare and \(ssg4\).
Supplemental Figure S3. Transmission electron microscope images of starch grains at DAF 5.

Supplemental Figure S4. Chloroplast morphologies in ssg4 flag leaves.

Supplemental Figure S5. SSG4 expression patterns in various tissues.

Supplemental Figure S6. Stroma-localization of SSG4N-GFP in chloroplasts.

Supplemental Figure S7. Accumulation of proteins involved in chloroplast division in ssg4 seeds at 7 DAF.

Supplemental Figure S8. Histograms showing the protein length distributions of DUF490-containing proteins.

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The authors would like to thank Dr. Shin-ichi Arimura (University of Tokyo) and Dr. Takehito Inaba (Miyazaki university) for providing us the plasmids, pL12-GFP and pCor413im1-GFP, respectively. We would also like to thank Mr. Makoto Kobayashi (RIKEN) and Prof. Kazuki Saito (RIKEN) for metabolite profiling analysis and Ms. Rie Hijiya (Okayama University) and the Biotechnology Center of Akita Prefectural University for their technical assistance.

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Figure legends

Figure 1. Enlarged starch grains (SGs) of matured endosperm in ssg4 mutant. (A, B) Nipponbare grains, front and side view images, respectively. Bars = 1 mm. (C, D) ssg4 grains, front and side view images, respectively. Bars = 1 mm. (E) Quantification of Nipponbare and ssg4 seed sizes (n = 30 each). (F, G) Iodine-stained thin sections of Nipponbare endosperm at low and high magnification, respectively. Bars = 10 μm. (H, I) Iodine-stained thin sections of ssg4 endosperm at low and high magnification, respectively. Bars = 10 μm. (J) Quantification of the areas occupied by SGs in sections of different genotypes (n = 6 each). (K) Quantification of the starch amount in matured seeds expressed as the percentage of the weight (n = 3 each). Data are given as the means ± SD. Statistical comparisons were performed using Welch’s t-test; all data were compared with Nipponbare (*P < 0.05, **P < 0.01).

Figure 2. Starch grains (SGs) in maturing endosperm. (A–C) Developing seeds of Nipponbare (NP) at 3, 5, and 7 days after flowering (DAF), respectively. Bars = 1 mm. (D–F) Developing seeds of ssg4 at 3, 5, and 7 DAF, respectively. Bars = 1 mm. (G–I) Iodine-stained thin sections of Nipponbare endosperm at 3, 5, and 7 DAF, respectively. Bars = 20 μm. (J–L) Iodine-stained thin sections of ssg4 endosperm at 3, 5, and 7 DAF, respectively. Bars = 20 μm. (M) Quantification of the areas occupied by SGs in sections...
at 3, 5, and 7 DAF (n = 20 each). (N) Quantification of the numbers of SGs per 10,000 μm² at 3, 5, and 7 DAF. Data are given as the means ± SD. Statistical comparisons were performed by Welch’s t-test; all data were compared with Nipponbare (**P < 0.01).

**Figure 3.** Starch morphologies in pollen grains, root caps and pericarp. (A, B) Iodine-stained pollen grains of Nipponbare and ssg4, respectively. Bars=10 μm. (C, D) Released starch grains (SGs) from squashed pollen grains of Nipponbare and ssg4, respectively. Bars = 10 μm. Insets show scanning electron micrographs of the released SGs. Bars = 1 μm. (E) Quantification of the areas occupied by SGs in pollen grains (n = 30 each). (F, G) Iodine-stained thin sections of root caps of Nipponbare and ssg4, respectively. Bars = 20 μm. (H, I) Magnified images of F and G. Bars = 20 μm. (J) Quantification of the areas occupied by SGs in root caps (n = 24 each). (K, L) Iodine-stained thin sections of pericarp in 3-DAF seeds of Nipponbare and ssg4, respectively. Bars = 10 μm. (M, N) Magnified images of K and L. Bars = 10 μm. (O) Quantification of the areas occupied by SGs in pericarps (n = 12 each). Data are given as the means ± SD. Statistical comparisons were performed by Welch’s t-test; all data were compared with Nipponbare (**P < 0.01).

**Figure 4.** Chloroplast morphologies in ssg4 third leaves. (A, B) Third leaves of Nipponbare and ssg4, respectively. Bars = 1 mm. (C, D) Thin-sections of the third leaves were double-stained with methylene blue and basic fuchsine; Nipponbare and ssg4, respectively. Bars = 10 μm. (E, F) Magnified images of C and D. Bars = 10 μm. (G)
Quantification of the areas occupied by chloroplasts in third leaves \((n = 12\) each). Data are given as the means ± SD. Statistical comparisons were performed by Welch’s \(t\)-test; all data were compared with Nipponbare (\(* * P < 0.01\)). (H, I) Transmission electron microscopic (TEM) images of chloroplasts of Nipponbare and \(ssg4\), respectively. Bars = 1 \(\mu m\). (J, K) TEM images of thylakoid and envelope membranes of Nipponbare and \(ssg4\), respectively. Bars = 200 nm.

**Figure 5.** Map-based cloning of \(SSG4\) gene. (A) Fine-mapping of the \(SSG4\) locus on Chromosome 1. A total of 229 F2 progenies (458 chromosomes) with homozygous \(ssg4\) alleles were analyzed. The numbers of recombinations that occurred between \(SSG4\) and the molecular markers are indicated. The \(SSG4\) locus was mapped to a 62 kb region between two molecular markers (Marker 1169 and Marker 13025). This region contains ten open reading frames (boxes). The \(ssg4\) mutant has a mutation in \(Os1g0179400\) (grey box). The position (4,133,703–4,140,631) is based on the Os-Nipponbare-Reference-IRGSP-1.0. (B) Schematic representation of the exon and intron organization of \(Os1g0179400\) and its cDNA obtained from RACE analysis. The deduced protein structure is also shown. The numbers in parentheses are the positions of chromosome 1 based on IRGSP-1.0. The \(ssg4\) mutant has a base-pair change (G to A) at position 4,139,234. Positions of primers (P1–P3) that were used for real-time PCR are indicated. The previously isolated cDNA clone (AK063507) covered approximately half of the full-length cDNA. \(SSG4\) encodes a protein containing 2,135 amino acid residues with a DUF490. Putative transit peptides (amino acids 1–42) and DUF490 (amino acids
1,730–2,119) are indicated by red and yellow boxes, respectively. The base-pair change in ssg4 causes an amino acid substitution at position 1,924 indicated by the red arrow.

(C) Phylogenic relationships of DUF490 sequences from bacteria to higher plants. Sequences are named by the GenBank/EMBL/DDBJ databases or UniProtKB identifications. SSG4 (Oryza sativa), BRAD12G05017 (Brachypodium distachyon), and DAA53165 (Zea mays) are monocot proteins. XP_002281904 (Vitis vinifera), AT2G25660 (Arabidopsis thaliana), and XP_003545508 (Glycine max) are dicot proteins. XP_002966241 (Selaginella moellendorffii) is from a pteridophyte; XP_001779881 (Physcomitrella patens) is from a bryophyte; CCO16912 (Bathycoccus prasinos) and Q016Y8 (Ostreococcus tauri) are from green algae; BAB74129 (Anabaena sp. PCC 7120) and P73551 (Synechocystis sp. PCC 6803) are cyanobacterial proteins; and E1WAU5 (Salmonella enterica), P39321 (Escherichia coli), and D2TN57 (Citrobacter rodentium) are proteobacterial proteins. Bootstrap values from 1000 trials are indicated. The 0.2 scale shows substitution distance. (D) Multiple amino acid sequence alignments of DUF490-containing proteins near the ssg4 mutation site. The alignment was produced with the Clustal W program using default parameters and was refined manually. Highly and moderately conserved residues are highlighted with green and yellow backgrounds, respectively. Different groups are shown by colored lines to the left of the protein names: red, monocot; blue, dicot; brown, pteridophyte; orange, bryophyte; green, green algae; violet, cyanobacteria; and black, proteobacteria. The glycine residue that was substituted with serine in ssg4 mutant is indicated by black arrowhead.
Figure 6. Complementation of ssg4 mutant with the genomic clone of the SSG4 gene. The genomic fragment including the SSG4 gene was expressed under the control of the maize ubiquitin 1 promoter in the ssg4 mutant background. Two independent transgenic plants (Ubi:SSG4genomic/ssg4 #5 and #8) were examined using the following tissues. (A–D) Mature endosperm. (E–H) Pollen grains. A and B are low magnification, C and D are high magnification. E and F are images of whole-mount pollen grains. G and H show SGs that were released from squashed pollen grains. Bars = 10 μm.

Table I. Effects of ssg4 mutation on the gelatinization properties of starch in endosperm determined by differential scanning calorimetry.

Gelatinization properties of the starch in ssg4 seeds were analysed by a differential scanning calorimeter. Values are means ± se of three independent determinations. $T_O$, $T_P$ and $T_C$ are onset, peak and conclusion temperatures, respectively. $\Delta H$ is gelatinization enthalpy of starch.

<table>
<thead>
<tr>
<th></th>
<th>$T_O$ (°C)</th>
<th>$T_P$ (°C)</th>
<th>$T_C$ (°C)</th>
<th>$\Delta H$ (mJ mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nipponbare</td>
<td>53.2 ± 2.6</td>
<td>63.2 ± 0.8</td>
<td>69.2 ± 0.5</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>ssg4</td>
<td>51.6 ± 2.1</td>
<td>61.6 ± 0.4</td>
<td>67.6 ± 0.8</td>
<td>5.8 ± 0.5</td>
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</table>
Table II. Segregation of ssg4 pollen grains of F1 plants.

Matured anthers from the F1 hybrid between ssg4 and Nipponbare were disrupted with forceps in the diluted Lugol solution on a glass slide to obtain the iodine-stained matured pollen grains. The released pollen grains were subsequently examined under the microscope.

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Number of WT pollen</th>
<th>Number of ssg4 pollen</th>
<th>Total</th>
<th>Percentage of ssg4 pollens</th>
</tr>
</thead>
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<tr>
<td>ssg4&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>59</td>
<td>58</td>
<td>117</td>
<td>50.4</td>
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</table>
Table III. Segregation of ssg4 seeds in F2 population.

F2 seeds were obtained from the cross between ssg4 and Nipponbare. Endosperm thin sections were prepared from 100 F2 seeds. The size of starch grains was examined under the microscope.

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Number of ssg4 seeds</th>
<th>Number of WT seeds</th>
<th>Total</th>
<th>$\chi^2$ value ($P$-value) for 1:3 segregation</th>
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<tbody>
<tr>
<td>$ssg4^{-/+}$</td>
<td>26</td>
<td>74</td>
<td>100</td>
<td>0.053 (0.82)</td>
</tr>
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</table>