GLUCAN SYNTHASE-LIKE 5 (GSL5) Plays an Essential Role in Male Fertility by Regulating Callose Metabolism During Microsporogenesis in Rice

Xiao Shi1,2, Xuehui Sun1,2, Zhiguo Zhang1, Dan Feng1, Qian Zhang1, Lida Han1, Jinxia Wu1,* and Tiegang Lu1**

1Biotechnology Research Institute/National Key Facility for Gene Resources and Gene Improvement, Chinese Academy of Agricultural Sciences, Beijing 100081, China
2These authors contributed equally to this work.

*Corresponding authors: Jinxia Wu, E-mail, wujinxia@caas.cn; Tiegang Lu, E-mail, lutiegang@caas.cn; Fax, +86-10-82106132.

(Received June 25, 2014; Accepted December 1, 2014)

Callose plays an important role in pollen development in flowering plants. In rice, 10 genes encoding putative callose synthases have been identified; however, none of them has been functionally characterized. In this study, a rice Glucan Synthase-Like 5 (GSL5) knock-out mutant was isolated that exhibited a severe reduction in fertility. Pollen viability tests indicated that the pollen of the mutant was abnormal while the embryo sac was normal. Further, GSL5-RNA interference transgenic plants phenocopied the gsl5 mutant. The RNA expression of GSL5 was found to be knocked out in the gsl5 mutant and knocked down in GSL5-RNA interference transgenic plants by real-time reverse transcription–PCR (RT–PCR) analysis. The male sterility of the mutant was due to abnormal microspore development; an analysis of paraffin sections of the mutant anthers at various developmental stages revealed that abnormal microspore development began in late meiosis. Both the knock-out and knock-down of GSL5 caused a lack of callose in the primary cell wall of meiocytes and in the cell plate of tetrads. As a result, the callose wall of the microspores was defective. This was demonstrated by aniline blue staining and an immunogold labeling assay; the microspores could not maintain their shape, leading to premature swelling and even collapsed microspores. These data suggest that the callose synthase encoded by GSL5 plays a vital role in microspore development during late meiosis and is essential for male fertility in rice.

Keywords: Callose • Male fertility • Microspore development • Pollen viability • Rice (Oryza sativa).

Abbreviations: CT, C-terminus; DAPI, 4',6-diamidino-2-phenylindole; FDA, fluorescein diacetate; GFP, green fluorescent protein; GSL5, Glucan Synthase-Like 5; GSL5-Ri, GSL5-RNA interference; GUS, β-glucuronidase; MMC, microspore mother cell; NT, N-terminus; PSSR, panicle seed setting rate; RT–PCR, reverse transcription–PCR; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TM, transmembrane domain; WT, wild type.

Introduction

Callose, a polysaccharide comprised mainly of β-1,3-glucan, provides structural support and protection to plant cells. The deposition of callose occurs in wide-ranging biological processes, from plant development to environmental stress responses (Jacobs et al. 2003, Chen and Kim 2009, Gupta et al. 2013, Eggert et al. 2014). Callose accumulates in the cell plate of dividing cells before the formation of the cell wall (Hong et al. 2001). During the plant stress response, callose accumulates at the inner surface of the wall adjacent to the plasma membrane (Enns et al. 2005, Jurgens 2005, Backues et al. 2007, Eggert et al. 2014). In addition, callose is present in phloem sieve plate pores, which are major channels that mediate the long-distance movement of signaling molecules and nutrients (Barratt et al. 2011). It has also been suggested that callose is located in the neck region of plasmodesmata where it regulates cell–cell communication (Lucas and Lee 2004, Vaten et al. 2011, Han et al. 2014).

In addition to the functions described above, callose plays a vital role in plant microsporogenesis. As shown in Supplementary Fig. S1, rice (Oryza sativa) pollen development is divided into 14 stages (D.B. Zhang et al. 2011). A temporary callose layer is formed between the primary cell wall and the plasma membrane, and it serves to separate the microspore mother cells (MMCs) prior to meiosis in the anther locule (Dong et al. 2005). During cytokinesis in microspores, callose deposition is transient and important for exine patterning. In the next stage, callose forms a temporary cell wall during the asymmetric division that splits the microspore into a large vegetative cell and a smaller generative cell (McCormick 1993, McCormick 2004, D.B. Zhang et al. 2011). The release of individual microspores requires co-ordinated callose synthesis and degradation (Izhar and Frankel 1971, Worrall et al. 1992). After pollen germination, pollen tube formation requires callose as a major component of the cell wall and plugs (Iglesias and Meins 2000, Bucher et al. 2001, Nishikawa et al. 2005). Thus, the timing of callose wall formation and degradation at several stages is critical for microspore development.

Callose is synthesized by a polysaccharide synthase, β-1,3-glucan synthase, using UDP-glucose as the substrate. Callose...
The multiple roles of callose deposition in microspore development have been revealed in several studies using callose synthase mutant plants. A number of studies have been conducted in *Saccharomyces cerevisiae*, *Brassica oleracea*, tobacco and other higher plants, but the most important studies have been conducted in Arabidopsis. Several *GSL* genes among the 12 *Glucan Synthase-Like* (*GSL*) family members in Arabidopsis are involved in callose deposition during microspore development. For example, *AtGSL2* (*CalS5*) is required for the formation of the cell wall surrounding the MMC, microspores and mature pollen, and it is required for the production of callose plugs and the tube wall during pollen tube growth (Dong et al. 2005, Nishikawa et al. 2013). *AtGSL10* (*CalS9*) is involved in pollen development at the mitotic division stage. The knock-out of *AtGSL10* leads to aberrant asymmetric microspore division (Huang et al. 2009). Further, *AtGSL1* (*CalS11*) and *AtGSL5* (*CalS12*) play partially redundant roles in sporophyte and pollen development. Both genes are responsible for the formation of the callose wall that separates the microspores of the tetrad, and they play a role in the gametophyte during pollen grain maturation (Enns et al. 2005). Moreover, *Atgsl8* (*calS10*) shows pleiotropic defects during embryogenesis and early vegetative growth (Chen et al. 2009). The role of callose in microspore development has been described in other plant species, including *B. oleracea*, tobacco and rice (Worrall et al. 1992, Elleman and Dickinson 1996, Wan et al. 2011).

Previously, a β-1,3-glucanase rice mutant, *osg1*, was isolated that exhibited the delayed release of young microspores into the anther locules due to disrupted callose degradation around the microspores (Wan et al. 2011). Abnormal callose deposition or degradation can lead to the formation of unnatural microspores. However, knowledge on callose synthesis during microsporogenesis in rice is limited.

In rice, there are 10 predicted genes belonging to the *GSL* family; however, none of them has been functionally characterized. To study the role of callose in microspore development, we screened a rice T-DNA insertion mutant library and isolated a microsporogenesis-related mutant, *gsl5*. Expression analysis of *GSL5* indicated that it is expressed in most plant tissues, with the highest levels of expression in tapetum, meiocytes, tetrads, microspores and mature pollen. The *gsl5* mutant and *GSL5*-RNA interference (*GSL5-Ri*) plants showed collapsed pollen grains that lacked a callose wall and exhibited an abnormal exine pattern. Our results demonstrate that *GSL5* plays a critical role in pollen wall formation and pollen viability.

### Results

#### Rice *gsl5* is a low-fertility mutant

To identify new genes involved in rice fertility, a rice T-DNA mutant library was screened for the phenotype of seed setting rate. A homozygous low fertility mutant was isolated with a panicule seed setting rate (PSSR) of 10.9% compared with 95.1% in the wild type (WT; *Fig. 1A, B*). The *gsl5* mutant was crossed to WT Nipponbare plants, and the observed spikelet fertility in the progeny reached 87%. Genetic analyses of heterozygous F1 plants showed that the *gsl5* phenotype segregated at a 3:1 ratio ($\chi^2 = 0.331 < \chi^2_{0.05, 1}$) in WT (214) and mutant-like (77) plants, indicating that the *gsl5* low PSSR phenotype was caused by a single recessive mutation. In addition, the mutant exhibited WT-like vegetative development (*Supplementary Fig. S2*). A PCR-based genome walking assay revealed that the T-DNA was inserted into the fifth intron of the *GSL5* gene in this low fertility mutant (*Fig. 1C*). An analysis of the mutant by real-time reverse transcription–PCR (RT–PCR) revealed that the insertion knocked out *GSL5* (*Fig. 1D*). To confirm that the *gsl5* mutation was responsible for the low fertility phenotype of the mutant, a *GSL5*-Ri vector was constructed and transformed into rice calli. All 32 independent T1 *GSL5*-Ri lines (they were independent in the T0 generation) were phenocopies of the *gsl5* T-DNA mutant; from these lines, three (*GSL5*-Ri-1, *GSL5*-Ri-2 and *GSL5*-Ri-4) were selected for further analysis. The PSSR in the three lines was 10.9, 14.3 and 12.5%, respectively (*Fig. 1A, B*). By real-time RT–PCR, the mRNA level of *GSL5* was found to be <20% of that in the WT (*Fig. 1D*).

### Characterization of *GSL5*

*GSL5* is composed of 40 exons spanning 16,696 bp and encoding 1,910 amino acid residues with a calculated molecular mass of 218 kDa (*Fig. 1C; Supplementary Fig. S4*). Multiple alignments were performed between 10 putative *GSL* from *O. sativa* and 12 *GSLs* from *Arabidopsis thaliana*. The ortholog of rice *GSL5* is *AtGSL2*, with 70.4% similarity in the full-length sequences (*Supplementary Fig. S3*). Amino acid sequence analyses indicated that members of the rice *GSL* family generally possess an FK51 domain (a large hydrophilic domain that is thought to provide catalytic activity) at the N-terminus (NT). The predicted topology of *GSL5* includes 14 transmembrane domains (TM) located at the NT (composed of eight TMs) and C-terminus (CT; composed of six TMs) (*Supplementary Fig. S4A, B*). Transient expression of *GSL5*-CT–green fluorescent protein (GFP) in rice protoplasts revealed that *GSL5* was localized to the plasma membrane (*Supplementary Fig. S4C*).

According to floret length, rice anther development can be divided into 14 stages (*Itoh et al. 2005, Wan et al. 2011, D.B. Zhang et al. 2011*). To examine the spatial and temporal expression of *GSL5* during anther development, real-time RT–PCR was conducted using a range of plant organs and tissues. The results indicate that *GSL5* was widely expressed in many of the tested organs and tissues, including leaves, stems and anthers. In particular, it was highly expressed in anthers at the following stages of development: the early meiosis (St7), later meiosis (St8) and early microspore (St9) stages (*Fig. 2A*). Transgenic plants carrying *GSL5 promoter-GUS* (pGSL5-GUS) showed *GSL5* promoter activity in rice anthers (*Fig. 2B, C*) and pollen grains (*Fig. 2D*). Meanwhile, an in situ hybridization analysis revealed the cellular specificity of *GSL5* expression in WT anthers. The most obvious signal was observed at the tapetum, but the signal was...
development, real-time RT–PCR was performed. The results show that GSL8 and GSL9 expression increased significantly, by >2-fold (Fig. 2I). The expression of GSL1, GSL2, GSL4 and GSL10 was also significantly increased (Fig. 2J).

**Male reproductive development was defective in the gsl5 mutant and GSL5-Ri plants**

To investigate the causes of low fertility in the gsl5 mutant, which exhibited normal growth at the vegetative stage, reproductive development was examined in the gsl5 mutant, GSL5-Ri transgenic and WT plants. Compared with WT plants, the gsl5 mutant and GSL5-Ri transgenic plants had shrunken anthers at the base of the locules, although the spikelets were normal in appearance (Fig. 3A, B). A normal embryo sac was observed in the gsl5 and GSL5-Ri plants by whole-mount stain-clearing confocal microscopy (Supplementary Fig. S5). To detect defects in anther dehiscence, an analysis of anthesis using the WT and gsl5 plants was performed. The results indicate normal dehiscence in the gsl5 anthers (Supplementary Fig. S6). Furthermore, we hand-pollinated the pistils of WT plants using pollen grains from the gsl5 mutant, and pistils of the gsl5 mutant plants using pollen grains from a WT plant. The PSSRs were 9.1% (\( \chi^2 = 3.2264 < \chi^2_{0.05,19} \)) and 82.7% (\( \chi^2 = 11.7337 < \chi^2_{0.05,19} \)), respectively. These data suggest that male sterility was the major cause of the low fertility observed in the gsl5 mutant.

**Microsporogenesis was compromised in the gsl5 mutant and GSL5-Ri plants**

According to the number of mature pollen grains per anther (264 ± 47 in the gsl5 mutant and 1,323 ± 144 in the WT), the total number of the gsl5 mutant pollen grains was 19.9% of the number in the WT. Most of the pollen grains in the gsl5 mutant were shrunken in appearance by scanning electron microscopy (SEM); however, 6.1% of the remaining mature gsl5 pollen grains were normal (Fig. 3C–F).

The KI–I2 staining rate (the number of stained pollen grains/the total number of mature pollen grains) was 5.1% in the gsl5 mutant, pollen fluorescein diacetate (FDA) staining and pollen germination analyses were performed. The FDA staining rate (the number of stained pollen grains/the total number of mature pollen grains) was 3.7% in the gsl5 mutant and 87.4% in the WT (Fig. 4B). The pollen germination rate was 3.0% and 85.9% in the gsl5 mutant and WT plants, respectively (Fig. 4C).

Similar to the gsl5 mutant, the KI–I2 staining rate (the number of stained pollen grains/ the total number of mature pollen grains) was 5.1% in the gsl5 mutant anthers. In contrast, the value was 97.2% in WT anthers (Fig. 4A). To investigate further the differences in the pollen grains between the WT and the gsl5 mutant, pollen fluorescein diacetate (FDA) staining and pollen germination analyses were performed. The FDA staining rate (the number of stained pollen grains/the total number of mature pollen grains) was 3.7% in the gsl5 mutant and 87.4% in the WT (Fig. 4B). The pollen germination rate was 3.0% and 85.9% in the gsl5 mutant and WT plants, respectively (Fig. 4C).

Similar to the gsl5 mutant, the KI–I2 staining rates in GSL5-Ri anthers were 6.9% in line GSL5-Ri-1, 6.1% in line GSL5-Ri-2 and 9.3% in line GSL5-Ri-4 (Fig. 4A); the pollen FDA staining ratios were 5.2% in GSL5-Ri-1, 4.4% in GSL5-Ri-2 and 5.9% in GSL5-Ri-4 (Fig. 4B). In addition, the pollen germination ratios were 3.8, 3.3 and 4.1% in lines GSL5-Ri-1, GSL5-Ri-2 and GSL5-Ri-4, respectively (Fig. 4C). However, a few viable pollen grains were detected in the gsl5 mutant and GSL5-Ri plants. Collectively, these data indicate that the male sterility we observed was due to a lack of viable pollen in the gsl5 mutant and GSL5-Ri plants.
Fig. 2 Specific expression of GSL5 in anthers. (A) Real-time RT–PCR analysis of GSL5 expression in various organs at different developmental stages. St, stage. Note that GSL5 was highly expressed in anthers in the early meiosis (St7), later meiosis (St8) and early microspore (St9) stages. (B–D) GUS staining of anthers from wild-type (WT) and transgenic plants expressing GSL5 promoter-GUS. (E–H) In situ hybridization analyses of GSL5 expression in the anthers of WT plants at (E and F) the tetrad stage and (G and H) mature pollen stage. (I) Real-time RT–PCR analysis of GSL5 expression revealed the knock-out and knock-down of the gene in the gsl5 and GSL5-Ri anthers, respectively, at different developmental stages. (J) Real-time RT–PCR analysis of these genes in the GSL family were conducted in the WT and gsl5 anthers at the late meiosis stage of microspore development. Error bars represent the SD of the mean of three biological replicates. Asterisks indicate significant differences according to Student’s t-test (\(*P<0.05\) and \(**P<0.01\)). SS, seedling stage; TS, tillering stage; MS, mature pollen stage; T, tapetum; Tds, tetrads; MP, mature pollen. Scale bars = 1 mm in (B and C), 30 μm in (D) and 100 μm in (E–H).
Abnormal tetrads and microspores in the gsl5 mutant

To investigate the role of GSL5 in microsporogenesis, we observed WT, gsl5 and GSL5-Ri anthers at eight developmental stages (Fig. 5A–X). No obvious difference was detected either in the MMC or in the four outer layers (the epidermis, endothecium, middle layer and tapetum) among the WT, the gsl5 mutant and GSL5-Ri-1 anthers prior to the tetrad stage (Fig. 5A–I).

At the tetrad stage, the cytoplasm of the tapetum was condensed and the haploid microspores in the tetrad were properly separated or enclosed by a callose wall in the WT anthers (Fig. 5J). In contrast, in the gsl5 and GSL5-Ri plants, the microspores of the tetrads were enlarged and abnormal in shape. A disordered border existed between the microspores, making them indistinguishable in the gsl5 mutant and GSL5-Ri-1 anthers (Fig. 5K, L). The released early microspores were spherical and the distribution of the cytoplasm was uniform and plump (Fig. 5M). In contrast, most of the microspores were deformed, with obvious swelling or shrinking, in the gsl5 mutant and GSL5-Ri-1 anthers (Fig. 5N, O). Ultimately, compared with the plump WT pollen (Fig. 5V), most of the pollen grains were collapsed in the gsl5 mutant and GSL5-Ri-1 plants (Fig. 5W, X). These data indicate that abnormal microspores emerged at the tetrad stage of anther development in the gsl5 mutant. Hence, GSL5 may function mainly during the tetrad stage.

Loss of callose at the microspore wall and cell plate

To investigate the involvement of GSL5 in callose synthesis during anther development, we monitored the callose level at different stages of microspore development. Aniline blue staining is commonly used to examine callose deposition during microspore development. In WT plants, the microspores displayed weak aniline blue fluorescence in the pollen mother cell (Fig. 6A). During meiosis, a callose wall surrounding the microspores was observed by aniline blue staining. A clear line showing a high density of aniline blue staining that separated the cells (i.e. a cell plate) was also observed (Fig. 6C). As the microspores developed to the tetrad stage, the cell plate developed a cross-wall that exhibited strong aniline blue staining. This signal was also detectable in the surrounding callose wall (Fig. 6F).

In the pollen mother cells of the gsl5 mutant, a normal aniline blue signal was detected (Fig. 6B). However, a loss of signal in the area surrounding the microspores at the dyad and tetrad stages was found in the gsl5 mutant and GSL5-Ri-1 plants, and virtually no callose wall was observed by bright field microscopy (Fig. 6D, E, G, H). Subsequently, a weaker or uneven aniline blue signal was observed in the cell plate of the gsl5 and GSL5-Ri-1 microspores. At the mature stage, the gsl5 pollen grains displayed a similar level of aniline blue staining to the WT. Thus, GSL5 is responsible for the synthesis of callose at the surface of microspores and cell plates. In addition, a 4',6-diamidino-2-phenylindole (DAPI) staining assay was performed and the results showed that meiosis was unaffected in the gsl5 mutant anthers (Fig. 6I–N; Supplementary Fig. S7).

Abnormal pollen exine wall in the gsl5 mutant

The consequence of reducing the amount of callose in the pollen grains was next examined at different stages of microspore development by transmission electron microscopy (TEM). The early microspore stage of anther development, a partial cavity emerged in the cytoplasm of most gsl5 mutant

Fig. 3 Compromised microsporogenesis in the gsl5 anther. (A) The gsl5 mutant has normal spikelets compared with the wild type (WT). le, lemma; pa, palea; gl, glume. Scale bar = 2 mm. (B) The gsl5 and GSL5-Ri anthers were defective. The basal part of the mutant anther was shriveled (arrowhead), and the length of the gsl5 mutant anther was only two-thirds of the length in the WT. Scale bar = 2 mm. (C–F) Scanning electron micrographs of pollen from WT (C) and gsl5 (D–F) mutant plants revealed aberrant phenotypes. Note the enlarged (D) and collapsed (E and F) gsl5 pollen grains. Bars = 10 μm.

Abnormal tetrads and microspores in the gsl5 mutant

To investigate the role of GSL5 in microsporogenesis, we observed WT, gsl5 and GSL5-Ri anthers at eight developmental stages (Fig. 5A–X). No obvious difference was detected either in the MMC or in the four outer layers (the epidermis, endothecium, middle layer and tapetum) among the WT, the gsl5 mutant and GSL5-Ri-1 anthers prior to the tetrad stage (Fig. 5A–I).

At the tetrad stage, the cytoplasm of the tapetum was condensed and the haploid microspores in the tetrad were properly separated or enclosed by a callose wall in the WT anthers (Fig. 5J). In contrast, in the gsl5 and GSL5-Ri plants, the microspores of the tetrads were enlarged and abnormal in shape. A disordered border existed between the microspores, making them indistinguishable in the gsl5 mutant and GSL5-Ri-1 anthers (Fig. 5K, L). The released early microspores were spherical and the distribution of the cytoplasm was uniform and plump (Fig. 5M). In contrast, most of the microspores were deformed, with obvious swelling or shrinking, in the gsl5 mutant and GSL5-Ri-1 anthers (Fig. 5N, O). Ultimately, compared with the plump WT pollen (Fig. 5V), most of the pollen grains were collapsed in the gsl5 mutant and GSL5-Ri-1 plants (Fig. 5W, X). These data indicate that abnormal microspores emerged at the tetrad stage of anther development in the gsl5 mutant. Hence, GSL5 may function mainly during the tetrad stage.

Loss of callose at the microspore wall and cell plate

To investigate the involvement of GSL5 in callose synthesis during anther development, we monitored the callose level at different stages of microspore development. Aniline blue staining is commonly used to examine callose deposition during microspore development. In WT plants, the microspores displayed weak aniline blue fluorescence in the pollen mother cell (Fig. 6A). During meiosis, a callose wall surrounding the microspores was observed by aniline blue staining. A clear line showing a high density of aniline blue staining that separated the cells (i.e. a cell plate) was also observed (Fig. 6C). As the microspores developed to the tetrad stage, the cell plate developed a cross-wall that exhibited strong aniline blue staining. This signal was also detectable in the surrounding callose wall (Fig. 6F).

In the pollen mother cells of the gsl5 mutant, a normal aniline blue signal was detected (Fig. 6B). However, a loss of signal in the area surrounding the microspores at the dyad and tetrad stages was found in the gsl5 mutant and GSL5-Ri-1 plants, and virtually no callose wall was observed by bright field microscopy (Fig. 6D, E, G, H). Subsequently, a weaker or uneven aniline blue signal was observed in the cell plate of the gsl5 and GSL5-Ri-1 microspores. At the mature stage, the gsl5 pollen grains displayed a similar level of aniline blue staining to the WT. Thus, GSL5 is responsible for the synthesis of callose at the surface of microspores and cell plates. In addition, a 4',6-diamidino-2-phenylindole (DAPI) staining assay was performed and the results showed that meiosis was unaffected in the gsl5 mutant anthers (Fig. 6I–N; Supplementary Fig. S7).

Abnormal pollen exine wall in the gsl5 mutant

The consequence of reducing the amount of callose in the pollen grains was next examined at different stages of microspore development by transmission electron microscopy (TEM). The early microspore stage of anther development, a partial cavity emerged in the cytoplasm of most gsl5 mutant
microspores (Fig. 7C, red arrowheads), while others developed an irregular shape (Fig. 7D, E). Sporopollenin deposition was observed at different stages of microspore development. It was detected as a high-density signal surrounding WT microspores by TEM. In contrast, irregular deposition of sporopollenin around the microspore was found in the shrunken microspores of the gsl5 mutant.

At maturity, most of the gsl5 pollen grains were abnormal in size or shape and exhibited a loss of starch granules compared with WT pollen (Fig. 7F–I). The shrunken pollen exhibited a
cystic structure; however, 6.1% of the remaining mature *gsl5* pollen grains displayed a normal shape and contained a nearly normal amount of starch granules. The pollen wall is composed of several layers, including a tectum, baculum, exine and intine. The exine can be divided into the sexine and nexine, whereas the nexine bilayer, consisting of a foot layer and endexine, is a simple layer that is laid down on the multilayered intine. The intine is the last layer of the pollen wall underlying the exine (Supplementary Fig. S9). In some of the *gsl5* mutant pollen grains, the exine layer of the pollen wall was expanded while the intine layer was reduced. In particular, the cells of the baculum were changed from columnar to granular in the *gsl5* mutant pollen (Fig. 7J–M).

**Discussion**

**GSLS may have redundant functions, but *GSL5* is critical for microgametogenesis**

*GSL5* is one of the 10 members of the rice *GSL* family, whereas 12 *GSL* genes exist in *Arabidopsis* (*AtGSL* genes). A number of *AtGSL* genes have already been identified and characterized; however, the *GSL* genes in rice have not yet been characterized. Callose synthesis in rice is controlled by 10 *GSL* genes, each of which is delicately regulated and displays a unique expression pattern. More importantly, GSLS may complement each other and work redundantly. The present study revealed that *GSL5* is expressed throughout anther development, with peak expression at stages 7–9 (Fig. 2A). In addition, most *GSL5* expression in anthers occurs in the tapetum, microspores and mature pollen (Fig. 2E–H). In this study, knock-out and knock-down plants of *GSL5* were isolated and characterized. *GSL5* encodes a protein with 1,910 amino acid residues. Like other GSLS, it was predicted to be a transmembrane protein with 14 transmembrane helices clustered into two segments by TMHMM (Supplementary Fig. S4A, B). The transient expression of *GSL5CT–GFP* in rice callus indicates that *GSL5* is mainly localized to the cell membrane (Supplementary Fig. S4C). The knock-out or knock-down of *GSL5* resulted in a lack of callose in the cell wall and cell plate of dyads and tetrads, and sterile pollen grains (Fig. 6D, E, G, H). Consistent with its function in microgametogenesis, *GSL5* is essential for callose deposition at the cell wall and the cell plate of dyads and tetrads during stages 7–9 of microspore development. Both the knock-out and knock-down of *GSL5* compromised microspore development at these stages, with a lack of callose in the cell wall and cell plate (Fig. 6D, E, G, H).

*Fig. 5* Arrested microspore development in wild-type (WT), *gsl5* and *GSL5-Ri-1* plants at the tetrad stage as seen by the staining of cross-sections (A–X) with 0.1% toluidine blue O. (A–C) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the pollen mother cell (PMC) stage. (D–F) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the early meiosis (EMe) stage. (G–I) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the mid-meiosis (MM) stage. (J–L) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the tetrad stage. (M–O) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the early microspore (Emi) stage. (P–R) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the late microspore (LM) stage. (S–U) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the mitosis stage. (V–X) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the mature pollen (MP) stage. Red arrowheads indicate abnormal tetrads, microspores and mature pollen grains. Scale bar = 15 μm.

*Fig. 5* Continued locules at the late microspore (LM) stage. (S–U) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the mitosis stage. (V–X) Cross-section of WT, *gsl5* and *GSL5-Ri-1* single anther locules at the mature pollen (MP) stage. Red arrowheads indicate abnormal tetrads, microspores and mature pollen grains. Scale bar = 15 μm.
Fig. 6 Aniline blue staining of microspore callose and DAPI staining of microspore nuclei during microsporogenesis, and immunogold labeling of cell wall and cell plate epitopes in tetrads. Aniline blue staining was applied to fixed reproductive tissues. Note that callose deposition was nearly 50%.

(continued)
Other GSL genes are also expressed in anthers, but their products cannot take the place of GSL5 as the major callose synthase in the cell wall and cell plate of microspores (Dong et al. 2005). However, the gsl5 mutant still had a PSSR of 10.9%, and the total number of the gsl5 mutant pollen grains was 19.9% of the number in the WT (Fig. 1A, B). Kl–I2 staining, FDA staining and pollen germination rate analyses indicated that a small portion of the pollen grains in the gsl5 mutant were still active (Fig. 4A–C). We also observed the induction of several GSLs in the gsl5 mutant (Fig. 2). These results suggest the expression of additional GSLs in anthers that play partially redundant roles with GSL5. Even in the collapsed gsl5 microspores, a small amount of callose remained at the cell plate and cell wall. This callose was synthesized by other GSLs and was less than the amount required for proper microspore development. In addition, according to our DAPI staining results, the gsl5 microspores proceeded successfully through meiosis, which requires accurate callose deposition at the cell plate (Fig. 6I–N). Therefore, GSL5 is a critical callose synthase for formation of the primary cell wall in microspores. In Arabidopsis, AtGSL2 is expressed in meiocytes, tetrads and microspores, with the highest level of expression in late meiosis and newly released microspores (Dong et al. 2005). Although megagametogenesis is normal in the gsl2 mutant, microspore development appears to be arrested (Dong et al. 2005). GSL5 exhibited a similar expression pattern in rice, and we found that abnormal microspores emerged at the tetrad stage during pollen development (Fig. 5J–L).

**Auxiliary role of GSL5 in other organs**

GSL5 was also found to be widely expressed in various rice organs, including root, leaf, sheath and stem (Fig. 2A). Information on the degree of redundancy between GSL5 and other GSLs in these organs is limited. Similar to Arabidopsis GSL genes, rice GSL genes could function independently with overlapping expression patterns. Most AtGSLs are broadly

---

**Fig. 6 Continued**

absent in the primary wall of the gsl5 microspores and reduced in the gsl5 dyads and tetrads. (A) Wild-type (WT) and (B) gsl5 mutant pollen mother cells. (C) WT, (D) gsl5 mutant and (E) GSL5-Ri-1 dyads. (F) WT, (G) gsl5 mutant and (H) GSL5-Ri-1 tetrads. (I–N) DAPI staining of WT (I–K) and gsl5 (L–N) microspores. (I and L) show microspores at the early meiosis stage, (J and M) show tetrads and (K and N) show newly released microspores. Scale bars = 50 μm in A–P and 30 μm in (Q–V). (O–R) Electron micrographs showing immunogold labeling of cell wall and cell plate epitopes in the WT (O and Q) and the gsl5 mutant (P and R) materials. CW, cell wall; CP, cytoplasm; Pm, plasma membrane; In, intine; Ex, exine; Ne, nexine; Ba, baculum; Fl, foot layer; Tc, tectum;Msp, microspore; T, tapetum.

**Fig. 7** Ultrastructures of mature pollen grains as shown by TEM. (A–E) Micrographs of newly released wild-type (WT; A) and gsl5 (B–E) pollen grains. Scale bars = 5 μm (A–D) or 10 μm (E). (F–I) Micrographs of mature WT (F) and gsl5 (G–I) pollen grains. Scale bars = 10 μm. (J–M) Micrographs showing the mature pollen exine structure in WT (J) and gsl5 (K–M) rice. Scale bars = 500 nm. Normal (C, G and K) and aberrant (D, E, H, I, L and M) pollen grains were observed in the gsl5 anther. Note that the cells in the baculum changed from columnar (J) to granular (L and M). Red arrowheads indicate abnormally deposited sporopollenin between the tectum and the foot layer in (L and M). St, starch granules; Cp, cytoplasm; Pm, plasma membrane; In, intine; Ex, exine; Ne, nexine; Ba, baculum; Fl, foot layer; Tc, tectum; Msp, microspore; T, tapetum.

505

---

expressed in a range of organs from roots to leaves, and the knock-out of different AtGSL genes results in defective development at diverse locations. AtGSL1 and AtGSL5 are partially redundantly involved in plant reproduction (Enns et al. 2005). AtGSL2 is responsible for pollen development and pollen tube growth (Nishikawa et al. 2005), while AtGSL5 encodes the enzyme responsible for callose synthesis at the cell membrane during the response to stress (Jacobs et al. 2003). AtGSL6 (CaGSL1) encodes a cell plate-specific callose synthase that interacts with two cell plate-associated proteins: phragmoplastin and a novel UDP-glucose transferase (Hong et al. 2001, Verma and Hong 2001). In the gsl7 mutant, callose is defective in the sieve plate pores of stems and roots (Barratt et al. 2011). The operation of an auxin–GSL8 feedback circuit controls the level of plasmodesmal-localized callose and down-regulates symplasmic permeability during the hypocotyl tropic response (Han et al. 2014). GSL8 also participates in pollen development, which requires the function of GSL10 (Toller et al. 2008). A gain-of-function mutation in gsl12 (CaGSL3) is identified that leads to a large amount of callose in the plasmodesmata of the mutant, which reduces intercellular trafficking (Vaten et al. 2011). Likewise, AtGSL2, an ortholog of rice GSL5, encodes a major callose synthase that plays the same role in the primary cell wall of microspores (Dong et al. 2005). Although GSL5 is widely expressed in various rice organs, the knock-out and knock-down of GSL5 resulted in normal vegetative growth (Supplementary Fig. S2). Given the reduced expression of GSL5 in root and leaf compared with floral tissue, the role of GSL5 in these organs might be auxiliary. However, the underlying evolutionary and regulatory mechanisms of the GSL family in rice are incompletely understood.

The timing of callose deposition and degradation is important for exine wall formation

During anther development, callose is synthesized and then deposited on the surface of the MMC before meiosis. As meiosis proceeds in microspores, dyads and tetrads come into being and are ultimately enclosed by a callose wall (Chen et al. 2009, Huang et al. 2009). The microspores of a dyad or tetrad are separated by a cell plate, which is composed largely of callose. Subsequently, the primexine appears on the outer surface of the microspores; the primexine acts as an elaborate template for the deposition of sporopollenin precursors (Li and Zhang 2010, Arizumi and Toriyama 2011). After meiosis, the callose wall is degraded and the microspores in the tetrads are released. The exine structure then continues to develop and the newly released microspores are enclosed by the proexine. Finally, stable microspores are formed through continuous thickening of the exine (Dong et al. 2005, Li and Zhang 2010).

In both the knock-out and knock-down mutants of GSL5, pollen mother cell development progressed normally. During meiois, the microspores of the gsl5 mutant exhibited WT characters before dyad or tetrad formation (Fig. 5A–I). At this stage, it is believed that callose forms a temporary wall to prevent cell cohesion and fusion (Enns et al. 2005). At the later meiosis stage, the callose wall not only separates the microspores, it also functions as a ‘molecular filter’ to protect the developing microspores from the influence of the surrounding diploid tissues (Heslop-Harrison and Mackenzie 1967) and as a physical barrier to prevent premature swelling and bursting of the microspores (Zhang et al. 2002). Due to the defective callose wall in the gsl5 mutant, the microspores were delicate and prone to shrinking or bursting. Later, the exine wall was defective due to the lack of sporopollenin and irregular exine, intine and baculum (Fig. 7D, E, H, I, L, M). Other GSLs are expressed during these stages of anther development; thus, a small amount of callose was detected. Nevertheless, the knock-out or knock-down of GSL5 resulted in imprecise callose deposition in terms of both timing and quantity. A similar phenomenon has been reported in the Arabidopsis mutant Atgsl2, which shows a defect in exine wall formation due to a reduction in callose deposition at the microspore cell wall (Dong et al. 2005). Collectively, these data suggest that callose synthesis is a critical factor that is carefully controlled to co-ordinate exine wall formation.

Materials and Methods

Plant materials and growth conditions

Rice (O. sativa ssp. japonica) cv. Nipponbare plants, the gsl5 mutant plants and GSL5-Ri transgenic plants were grown in a greenhouse at 30°C (16 h of light) and 22°C (8 h of dark) or in a paddy field at the Chinese Academy of Agricultural Sciences in Beijing, China, from May to October of each year.

Genetic analysis and PCR walking

Heterozygous plants were crossed with Nipponbare. A co-segregation assay was performed to detect the correspondence between the phenotype and the gene in the F1 generation, and a homozygous low-fertility mutant was obtained in the T1 generation. Genomic DNA flanking the T-DNA left border was cloned using PCR walking with nested specific primers (Zou et al. 2011; Supplementary Table S1).

Generation of GSL5-Ri transgenic rice plants

To construct the RNAi vector, the 720 bp cDNA sequence (including 60 bp of 3’ coding sequence and 660 bp of 3’ non-coding sequence) of GSL5 was amplified and inserted into pTCK300. Agrobacterium tumefaciens-mediated rice callus transformation was performed according to the method of Yang (2004). The primer sequences are listed in Supplementary Table S1.

Evaluation of pollen, embryo sacs and spikelet fertility

To investigate the viability of the pollen in the gsl5 mutant, 10 spikes from different mutant and WT plants were collected shortly before anthesis and fixed in Carnoy’s fluid. The samples, 20 pollen anthers from the WT and the gsl5 mutant plants, respectively, were handled with tweezers to squeeze out the pollen grains for staining with 1% KI–I2 and inspection by light microscopy. The same technique was used to gather pollen for staining with 10 µg ml−1 FDA (Sigma-Aldrich, http://www.sigmaaldrich.com) and observation by fluorescence microscopy. For the pollen germination assay, we used liquid medium [20% sucrose, 10% polyethylene glycol (PEG) 4000, 3 mM KCl, 1 mM calcium nitrate, 40 mg l−1 boric acid and 10 mg l−1 vitamin B1] (Wang et al. 2000). One or two drops of medium were placed on a clean slide, and then the anthers were added to the medium and squeezed to release the pollen. After covering with a cover glass, the slides were cultured for 30 min at 28°C in the dark. The pollen grains were then observed under a microscope. Pollen grains with a pollen tube that...
was longer than the diameter of the pollen grains were scored as having germinated successfully.

Whole-mount stain-cleaning laser scanning confocal microscopy (Liu et al. 2004, Watanabe et al. 2005, Zhao et al. 2007) was used to evaluate the structure of mature embryo sacs from WT and the gsl5 mutant plants. More than 100 mature florets from WT and the gsl5 mutant plants were fixed in FAA solution containing an 18 : 1 : 1 (by vol.) mixture of formalin, 70% ethanol and acetic acid. The solutions were then placed in a vacuum for 30 min and incubated for 24 h at room temperature, after which the tissue was stored in 70% ethanol at 4 °C. Before staining, the samples were transferred to 70% ethanol, removing the seed coat and staining the ovary tissue with 2% (w/v) uranyl acetate and 2.6% (w/v) lead citrate. Samples were then dehydrated by passing through an ethanol series (50, 30 and 15%) and finally placed in distilled water. The whole mature ovary was incised in 2% potassium (Sigma-Aldrich) sulfate for 20 min, held in an Eosin Y (Sigma-Aldrich) water solution for 10–12 h, placed in 2% potassium sulfate solution once again for 20 min, washed with distilled water, kept in it for 24 h, and finally washed three or four times in tap water. The samples were dehydrated by passing through an ethanol series (30, 50, 70 and 100%) and cleared by immersing them three times in 100% methyl salicylate (Sigma-Aldrich) (for > 10 h during the final incubation). The fertility of the embryo sacs was examined by confocal laser scanning microscopy.

The PSSR was calculated based on the number of seeds set per spikelet on each panicle at maturity. More than 20 panicles of the gsl5 mutant were bagged before flowering and hand pollinated with mature WT pollen every day for 3 d. One month later, the PSSR in the bagged spikelets was calculated.

In situ hybridization

Tissue preparation, in situ hybridization and immunodetection were performed as described previously (Xue et al. 2008). The GSL5 probe was PCR-amplified using gene-specific primers (GSL5-sensep-F and GSL5-sensep-R; Supplementary Table S1) to amplify the sense probe and a different set of gene-specific primers (GSL5-antisensep-F and GSL5-antisensep-R; Supplementary Table S1) to amplify the antisense probe. Next, the sense and antisense probes were each transcribed in vitro by T7 transcriptase, using a DIG RNA labeling kit (Roche, http://www.roche.com.cn). Images were obtained using a fluorescence microscope (Zeiss AXIO Imager A1, http://www.zeiss.com.cn).

GSL5–GUS reporter gene construction and analysis

To construct GSL5 promoter::GUS, a 2.1 kb fragment of the GSL5 promoter (~2,197 to –31 bp from the ATG) was amplified using the primers Pgsl5gusF and Pgsl5gusR (Supplementary Table S1) from genomic DNA and inserted into pCAMBIA1391Z. Transgenic plants were generated as described above. β-Glucuronidase (GUS) histochemical staining was performed as described previously (Sun et al. 2014).

Subcellular localization

To construct pANS80–GSL5–CCT-eGFP, the 1,041 bp coding sequence of GSL5 (4,287 to 5,328 bp from the ATG; including nine C-terminal TMs) was amplified using the primers pAN580P1F and pAN580P1R (Supplementary Table S1) from cDNA and fused into pAN580. The vectors pANS80–GSL5–CCT-eGFP and pANS80–mCherry-SAG29 were co-transfected into rice protoplasts according to published protocols (Y. Zhang et al. 2011). The plasmid mCherry-SAG29, which contains an endoplasmic reticulum retrieval tetrapeptide, was used as a membrane marker (Chiu et al. 1996, Nelson et al. 2007, Mao et al. 2012). The samples were observed with a confocal laser scanning microscope (Leica Microsystems TCS SP5, http://www.leica-microsystems.com).

Real-time RT–PCR

For the expression analysis of GSL5, total RNA was extracted using TRIzol solution (Invitrogen, http://www.invitrogen.com) from different tissues and florets at different developmental stages. Total RNA from each sample was reverse-transcribed with oligo(dT) primer and PrimeScript RT Enzyme (TAKARA, http://www.takara.com.cn) according to the manufacturer’s instructions. Real-time RT–PCR was conducted using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, http://www.bio-rad.com/) with three biological repeats. The rice Actin gene was used as an internal control to normalize the expression of GSL5. The gene-specific primers used for quantitative RT–PCR are listed in Supplementary Table S1.

Histological analysis

Spikelets at different developmental stages were collected based on their length and fixed in Carnoy’s fluid (containing a 3 : 1 (v/v) mixture of 100% ethanol and acetic acid). After treatment with 70, 83, 95 and 100% ethanol for 1 h, the samples were immersed in xylene for 2 h and then embedded in Paraplast Plus (Sigma-Aldrich). Microtome (RM2155; Leica Microsystems) sections (8 μm thick) were stained with 0.1% toluidine blue O (Sigma-Aldrich) for 6–8 min, and then the samples were hydrated using a graded ethanol series. The samples were then inspected using a microscope (Zeiss).

For callose staining, the samples were transferred to 70% ethanol, removing the seed coat and staining the ovary tissue with 2% (w/v) uranyl acetate and 2.6% (w/v) lead citrate. The pollen grains were then stained with 0.01% aniline blue (Sigma-Aldrich) (Hong et al. 2001, Peng et al. 2013).

Immunolocalization of rice tetrad cell wall and cell plate epitopes

Anthers (3–3.5 mm in length) from WT and the gsl5 mutant materials were fixed in 3.0% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.2 N sodium phosphate buffer, pH 7.0, and then post-fixed in 2% OsO4 in phosphate-buffered saline (PBS), pH 7.2, for 1 week. The samples were then treated as described previously (Lam et al. 2001, Maeda et al. 2006). The presence or absence of callose was determined using immunolocalization and TEM with monoclonal antibodies to β-1,3-glucan (Biosupplies, http://www.biosupplies.com.au). The primary and secondary antibodies (18 nm antimouse IgG–gold conjugate; Jackson ImmunoResearch, https://www.jacksonimmuno.com/) were used at dilutions of 1 : 100 and 1 : 20, respectively.

SEM and TEM

For SEM, fresh pollen grains were coated with 8 nm gold particles and observed using a JSM-6400 microscope (S3400N; Hitachi Ltd., http://www.hitachi.com/). For TEM, samples were fixed in 3.0% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.2 N sodium phosphate buffer, pH 7.0, and then post-fixed in 2% OsO4 in PBS, pH 7.2. Following dehydration in an ethanol series, the samples were embedded in acrylic resin. Ultrathin sections (50–70 nm) were double-stained with 2% (w/v) uranyl acetate and 2.6% (w/v) lead citrate and examined with a JEOL 1230 transmission electron microscope (JEOL Ltd., http://www.jeol.co.jp/en/) at 80 kV.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the China Rice Functional Genomics Project [project No. 2012AA10A304]; the International Science and Technology Collaboration Project [project No. 2014DFA31550].

Acknowledgments

We thank Dr. Jianmin Wan (ICS, CAAS) for the pmtCherry-SAG29 and pANS80 plasmids.
Disclosures

The authors have no conflicts of interest to declare.

References


Engreved GFP as a vital reporter in plants. Plant Physiol. 146: 505–511.


X. Shi et al. | GSL5 plays a key role in microspore development


