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Callose synthase (CalS5) is required for exine formation during microgametogenesis and for pollen viability in Arabidopsis

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Summary

Callose (β-1,3-glucan) is produced at different locations in response to biotic and abiotic cues. Arabidopsis contains 12 genes encoding callose synthase (*CalS*). We demonstrate that one of these genes, *CalS5*, encodes a callose synthase which is responsible for the synthesis of callose deposited at the primary cell wall of meiocytes, tetrads and microspores, and the expression of this gene is essential for exine formation in pollen wall. *CalS5* encodes a transmembrane protein of 1923 amino acid residues with a molecular mass of 220 kDa. Knockout mutations of the *CalS5* gene by T-DNA insertion resulted in a severe reduction in fertility. The reduced fertility in the *cals5* mutants is attributed to the degeneration of microspores. However, megagametogenesis is not affected and the female gametes are completely fertile in *cals5* mutants. The *CalS5* gene is also expressed in other organs with the highest expression in meiocytes, tetrads, microspores and mature pollen. Callose deposition in the *cals5* mutant was nearly completely lacking, suggesting that this gene is essential for the synthesis of callose in these tissues. As a result, the pollen exine wall was not formed properly, affecting the baculae and tectum structure and tryphine was deposited randomly as globular structures. These data suggest that callose synthesis has a vital function in building a properly sculpted exine, the integrity of which is essential for pollen viability.

Keywords: callose, male sterility, exine wall, microsporogenesis, pollen development.

Introduction

In developing anthers of angiosperms, microsporocytes synthesize a specialized temporary cell wall consisting of callose (a β-1,3-linked glucan) between the primary cell wall and the plasma membrane. Callose continues to be deposited through both meiotic divisions and it eventually encloses the microspore tetrad in a thick callose wall. Multiple roles of this temporary wall have been proposed over the last 40 years. It is believed that the callose layer is formed temporarily to prevent cell cohesion and fusion, and upon its degradation it facilitates the release of free microspores into the locular space (Waterkeyn, 1962). The callose wall may also function as a 'molecular filter' protecting the developing microspores from the influence of the surrounding diploid tissues (Heslop-Harrison and Mackenzie, 1967). It also provides a physical barrier that may help prevent premature swelling and bursting of the microspores. The callose wall appears to act as a mold wherein the primexine provides a blueprint for the formation of exine pattern on the mature pollen grain soon after the completion of meiosis during microsporogenesis (Stanley and Linskens, 1974; Waterkeyn and Beinfait, 1970; Zhang *et al.*, 2002).

Following tetrad formation, exine synthesis begins in the microspore. During this process, a β-1,3-glucanase (callase) is secreted by the tapetum cells and released into the locular space (Steiglitz, 1977). Callase activity in the anthers is low during the first meiotic division but increases rapidly at the end of the second meiotic division. Finally, the callose wall is degraded and microspores are released in the locular space (Frankel *et al.*, 1969; Steiglitz, 1977; Steiglitz and Stern, 1973). Several mutants in the callose wall formation and

dissolution have been characterized in Petunia, and it has been suggested that the timing of callose wall formation and degradation is pivotal for the normal development of the pollen (Izhar and Frankel, 1971; Warmke and Overman, 1972). In transgenic tobacco expressing a β-1,3-glucanase in tapetum cells, the callose wall is dissolved prematurely affecting pollen development resulting in male sterility (Worrall *et al.*, 1992).

Numerous studies have described pollen wall development and exine formation (Blackmore and Barnes, 1990; Scott, 1994; Stanley and Linskens, 1974; Yui *et al.*, 2003; Zinkl *et al.*, 1999). The exine pattern is highly species-specific and can be considered as a 'signature' of a species. Exine is divided into two main layers: an outer sculpted layer, the sexine, and an inner layer, the nexine (Horner Jr, 1977; Stanley and Linskens, 1974). The exine is mainly composed of sporopollenin, an aliphatic polymer (Ahlers *et al.*, 1999; Hernandez-Pinzon *et al.*, 1999; Meuter-Gerhards *et al.*, 1999; Millar *et al.*, 1998; Mou *et al.*, 2000), the composition of which may vary between different species. Although this complex material is synthesized by microspore and tapetum, how it is deposited in a species-specific pattern in the pollen is not well understood. Furthermore, factors that determine exine patterning have not been fully elaborated.

Several Arabidopsis mutants defective in exine formation have been isolated and characterized. These include *defective in exine formation 1* (*dex1*), *male sterility 2* (*ms2*), *faceless pollen-1* (*flp1*), and *no exine formation* (*nef1*). Sporopollenin is deposited randomly on the plasma membrane of *dex1* microspores and pollen grains are degraded. DEX1 protein could be a component of either the primexine matrix or endoplasmic reticulum and may participate in the assembly of primexine precursors (Paxson-Sowders *et al.*, 2001). In the *ms2* mutant, the exine wall is thin and sensitive to acetolysis treatment. The MS2 protein is a fatty acyl reductase that reduces long-chain fatty acids to fatty alcohol, one of the reactions required for the formation of sporopollenin (Aarts *et al.*, 1997). In the *flp1* mutant, the microspores and their exine are visually normal, but the exine pattern is sensitive to acetolysis. FLP1 protein appears to be a transporter or a catalytic enzyme involved in fatty acid biosynthesis, which is a necessary step for the synthesis of sporopollenin and wax crystals (Ariizumi *et al.*, 2003). In *nef1* mutant, sporopollenin is synthesized but is deposited in the locular wall. NEF1 is an integral membrane protein in the plastids of the tapetum cells where lipid synthesis takes place (Ariizumi *et al.*, 2004). No abnormality in callose synthesis during microsporogenesis was observed in these four exine-defective mutants.

Our results demonstrate that the *CalS5* (*Gsl2*) gene is responsible for the synthesis of callose in the temporary callose wall of the microspores and is essential for exine formation during microsporogenesis in Arabidopsis. To study the role of callose in exine formation, we have isolated and characterized T-DNA insertional mutants in the *CalS5* gene (*cals5*). The *cals5* mutant exhibited male sterility and lacked the normal callose wall affecting exine pattern of the microspores. Tryphine was synthesized but was randomly deposited as aggregates on the surface of microspores. Upon release from the tetrad, microspores did not survive and the pollen wall collapsed. This demonstrated that callose plays a key role in the synthesis of exine of pollen wall and the viability of the pollen.

Results

Isolation of cals5 *T-DNA knockout mutants*

We had earlier identified 12 *CalS* (*Gsl*) genes in Arabidopsis functionally and classified this family (Hong *et al.*, 2001a). To investigate possible roles of the different *CalS* genes in plant development, a collection of the Salk Arabidopsis T-DNA lines was screened that contain an insertion in these genes. We found two independent insertion lines that exhibited severely reduced silique length and seed yield in otherwise apparently 'normal' plants. The two lines, Salk_009234 and Salk_026354, contain a T-DNA insertion in intron 15 of the *CalS5* gene. Their homozygous lines are referred as *cals5-1* and *cals5-2*. To identify a homozygous T-DNA plant, genomic DNA was isolated from leaves of individual plants of each line. Using two *CalS5* gene-specific primers and a T-DNA primer (Figure 1a), PCR analysis of the genomic DNAs was carried out. The wild-type plants produced two bands of 907 and 971 bp, while the genomic DNAs of homozygous T-DNA insertion plant generated a PCR product of about 600 bp for *cals5-1* and a 650 bp product for *cals5-2* (Figure 1b,c). Complete knockout of the *CalS5* gene expression in these homozygous lines was verified by the absence of *CalS5* RNA transcripts using RT-PCR. For this purpose, total RNA was isolated from inflorescence of the wild type and mutant plants. RNA from the wild-type plants produced a PCR band of approximately 1200 bp, whereas RNA from the knockout plants failed to produce any PCR product (Figure 1d). Homozygous *cals5-1* mutant was backcrossed with the wild-type plant to check co-segregation of the phenotype. Anthers from the F_1 plants were dissected and 200 pollen tetrads were recorded. The ratio between the normal and shrunken pollen grains was found to be 1:1 (Figure 2h,i). Aniline blue staining for callose was very weak in the shrunken pollen grains when compared with that in normal pollen grains (Figure 2h,i).

CalS5 is a transmembrane protein and contains multiple functional motifs

The predicted coding region of *CalS5* gene is composed of 39 exons (Figure 1a) and encodes a peptide of 1923 amino acid residues with a calculated molecular mass of 220 kDa.

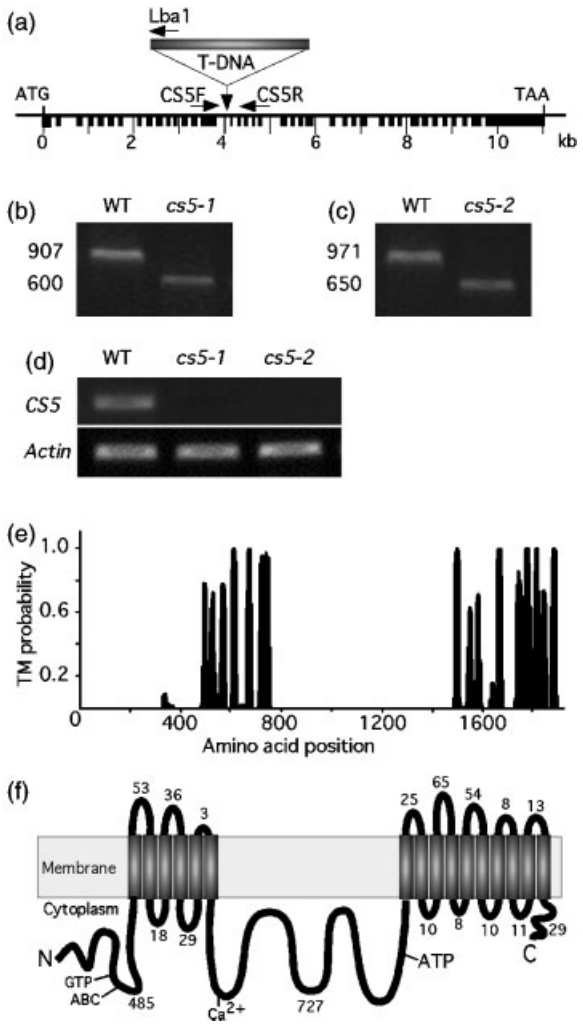


Figure 1. Identification of T-DNA insertion knockout lines of the *CalS5* gene.
(a) CalS5 gene (thin line) contains 39 exons (thick lines) in the coding region. Salk_009234 and Salk_026354 lines have a T-DNA inserted in the *CalS5* gene. Primers used to identify the homozygous line were marked by arrows. PCR products (907 and 600 bp for *CalS5-1*, and 971 and 650 bp for *CalS5-2*) of genomic DNA from wild type (WT) and homozygous T-DNA insertional lines, respectively, are indicated.
(b, c) PCR products of genomic DNA from WT and homozygous T-DNA lines of *cals5-1* (*cs5-1*) and *cals5-2* (*cs5-2*).
(d) RT-PCR of total RNA from WT and T-DNA lines. A PCR product of 1200 bp was produced when total RNA from wild-type plants was used, whereas no RT-PCR product was detected in the T-DNA lines. The expression of *Actin-2* gene served as control.
(e) Transmembrane (TM) probability of CalS5 peptide predicted by the transmembrane hidden Markov model (TMHMM) program.
(f) Topology of CalS5 in membrane. The long rectangle indicates the membrane and the vertical black bars represent the transmembrane helices of CalS5. The length of the peptide chain in each non-membrane-spanning segment is indicated by the number of amino acid residues.

Topology analysis of CalS5 revealed that, similar to CalS1 (Hong *et al.*, 2001a), it is a transmembrane protein with 16 transmembrane helices that are clustered in two regions separated by a large hydrophilic domain (727 residues). The hydrophilic N-terminus (485 residues) of CalS5 contains a GTP-binding motif (Prosite PDOC00185) and an ABC transporter motif (Prosite PDOC00185; Figure 1f). The central hydrophilic loop contains a calcium-binding domain (Prosite PDOC00018) and a β-1,3-glucan synthase component that is conserved in the glycosyltransferase 48 family that includes β-1,3-glucan synthases from yeast and plants (Verma and Hong, 2001). Thus, the overall structure of this gene is very similar to those encoding other callose synthases but its expression is regulated in a pollen-specific manner. That *CalS5* is a functional gene was demonstrated by RT-PCR using RNA from flower buds and specific primers which yielded four fragments of cDNA (1–1.5 kb each) corresponding to the predicted CalS5 cDNA sequence (data not shown).

Mutation in CalS5 *causes pollen sterility*

There were no observable differences in the vegetative parts of the *cals5* mutant and wild-type plants. They had comparable growth rates and leaf numbers throughout the vegetative growth and were similar in plant size (Figure 2a). However, the mutant plants developed abnormal flowers with shrunken anthers (Figure 2e,g) and were partially or completely sterile. Siliques formed on the homozygous *cals5* mutants were very short and contained no or only few seeds, when compared with those on the wild-type plants (Figure 2c). Flowers of the mutant plants were open for considerably longer periods than those of the wild-type plants, and many remained unfertilized. The flowers were completely fertile and developed normal-size siliques if cross-pollinated with the wild-type pollen grains (see below), suggesting that the female sex organs of the mutant plants were normal. Thus, *CalS5* gene appears to specifically affect microgametophytic development.

Effect of CalS5 *mutation on tetrad formation and pollen development during male gametogenesis*

The defects in pollen formation and fertility led us to analyze in more detail the development of male and female gametophytes. Both wild type and *cals5* mutant developed similar-size meiocytes in their anthers (Figure 3a,b). However, the tetrads, the products of meiosis, were abnormal in the *cals5* mutant plants. Under light microscope, the borders between the four cells of a tetrad became undistinguishable (Figure 3c,d) suggesting that the callose cell wall may not be formed. Microspores of the mutant plants exhibited severe morphological defects (Figure 3e,f). They were shrunken, broken, and eventually degenerated (Figure 3g,h). As a result, no viable pollen was developed in most of the mature anthers of the mutant plants. A few anthers were able to develop 1–15 mature pollen that were viable and fertile, whereas a normal anther of the wild-type plant may contain up to 800 mature pollen grains.

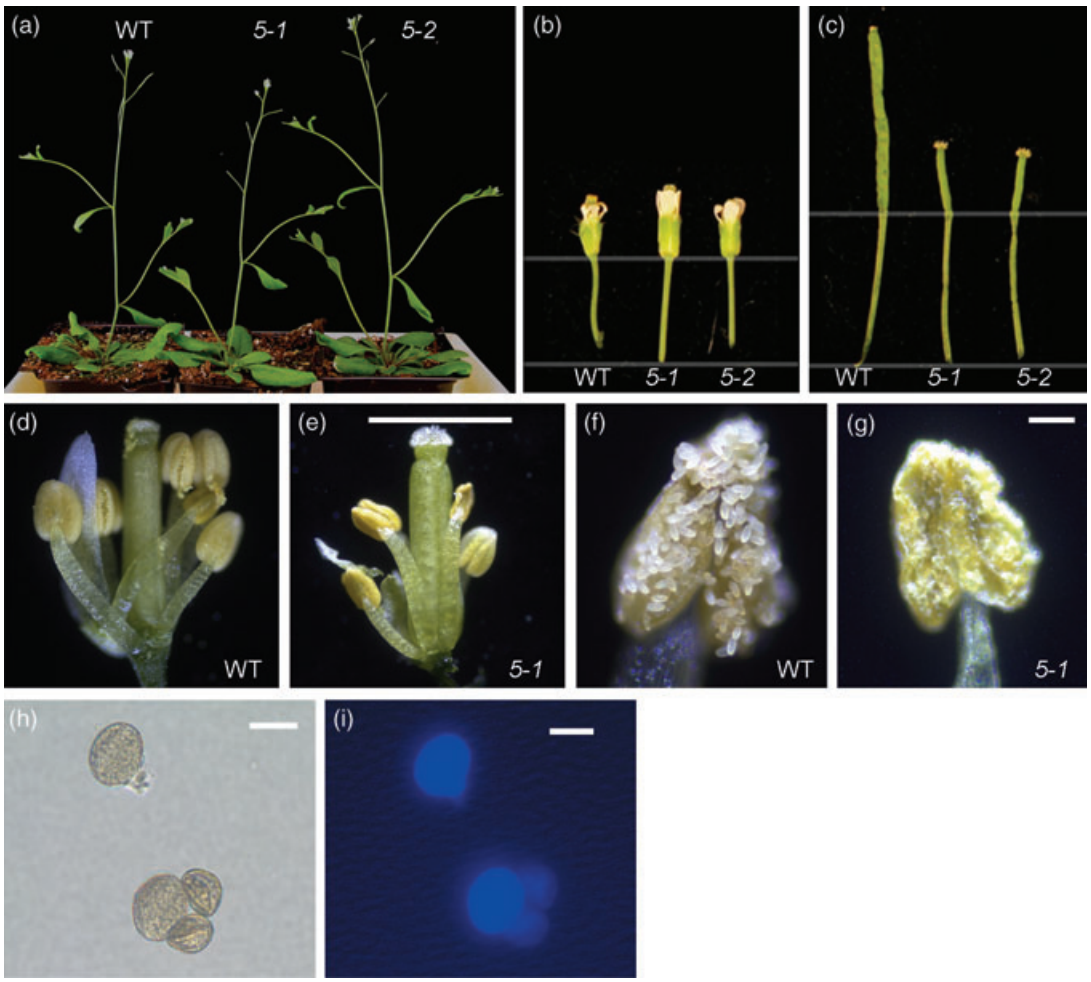


Figure 2. Reduction in fertility in *cals5* mutants.
(a) No apparent morphological defects except siliques were found in *cals5-1* (*5-1*) and *cals5-2* (*5-2*) mutants when compared with wild-type (WT) plants.
(b, c) Comparison of flowers and mature siliques between the wild type and *cals5* mutant lines. Note that the length of flower stalks is similar but that of the siliques is different between the wild type and mutants.
(d, e) Comparison of mutant and wild-type flowers. Note that anthers of the *cals5-1* mutant line are shrunken. Bar = 50 μm.
(f, g) Comparison of mutant and wild-type anthers. Note that anthers of the *cals5-1* mutant line contain few pollen grains. Bar = 50 μm.
(h, i) Cosegregation of shrunken pollen with the callose synthesis defect in F_1 pollen. Note that tetrads of microspores show two normal and two shrunken pollen. Bar = 10 μm.

Megasporogenesis and megagametogenesis in cals5 *mutant plants are not affected*

Megasporocytes in *cals5* mutants undergo normal cell divisions during early ovule development as observed in wild-type plants (Figure 3i,l). After the first two mitotic divisions of the functional megaspore during megagametogenesis, the developing embryo sac contained four nuclei that follow a final mitotic division and led to the eight nuclei contained in the mature gametophyte. One visible central cell in the embryo sac eventually developed at the late stage during megagametogenesis (Figure 3j,l). No apparent differences in megasporogenesis and megagametogenesis between the *cals5* mutant and the wild-type plants were observed, suggesting ovule development in the *cals5* mutants is not affected. When cross-pollination with the wild-type Arabidopsis pollen was performed, the *cals5* mutant flowers developed normal-size siliques which contained an average of approximately 40 seeds per silique, which was comparable with 42 seeds per silique recovered on the wild-type plants under the same growth conditions. This showed that both ovule development and female fertility in the *cals5* mutants are normal and that sterility is solely due to the defective male gametes.

Lack of viable pollen grains in cals5 *mutants*

Mutant plants with a severe sterility phenotype had small brown recessed anthers that appeared to lack pollen grains. Observed under differential interference contrast (DIC)

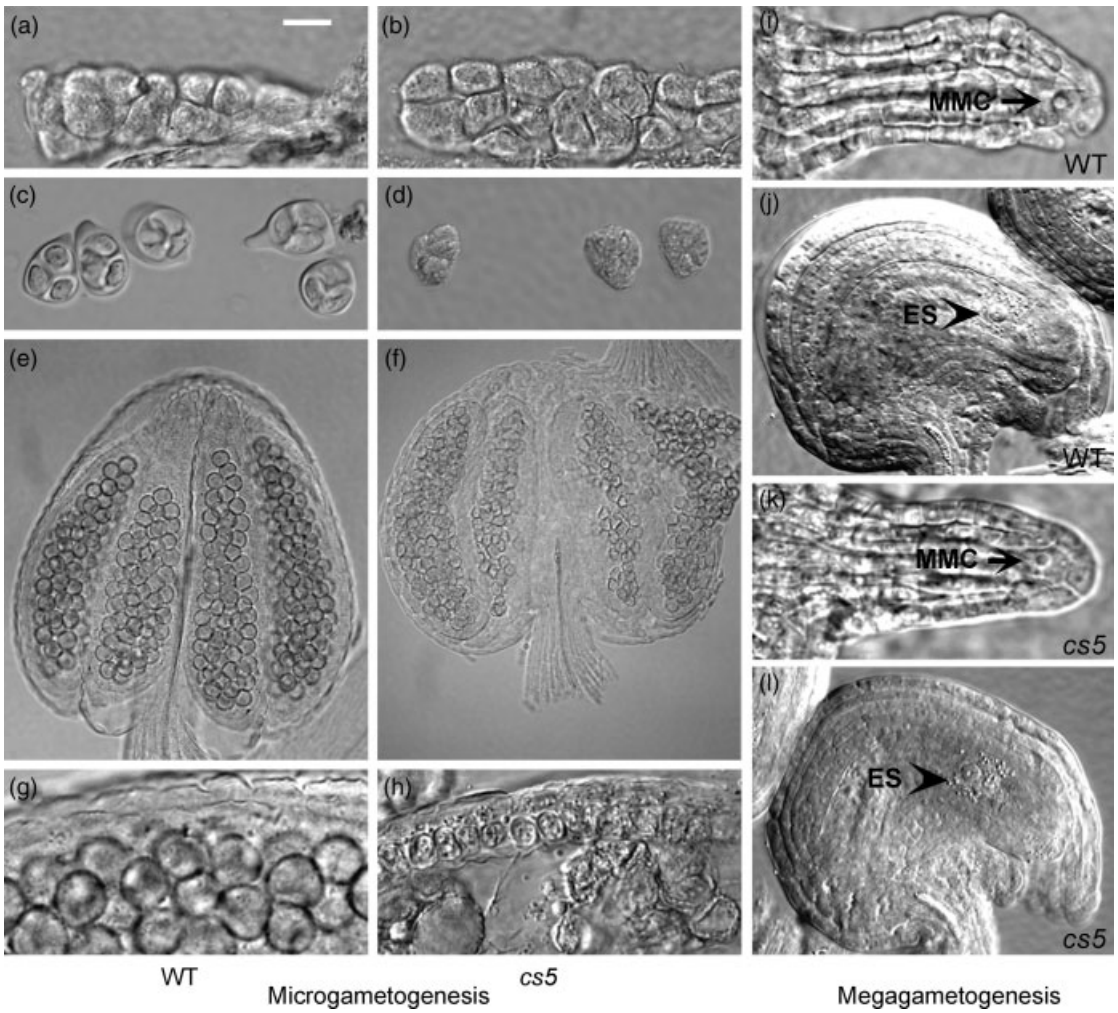


Figure 3. Male and female gametophyte development in *cals5* mutant lines.
(a, b) Developing pollen mother cells shows uniform meiocytes in the wild type (a) and mutant plants (b). Bar = 10 μm.
(c, d) Tetrad of microspores, products of meiosis. The microspores of wild-type plants are encased in callose walls and are separated by clear boundaries. In the mutants, the four microspores form a clump and the boundaries are barely recognizable. Bar = 10 μm.
(e, f) Comparison of microspores. After the release from the tetrad, microspores continue to develop into round and mature pollen grains in the wild-type plants, whereas they start to deform and degenerate in the mutants. Bar = 50 μm.
(g, h) Enlarged views of the developed microspores in wild-type plants and the degenerated microspores in the mutants. Bar = 50 μm.
(i–l) Megaspore mother cell (MMC, arrow) of the wild type (i) and mutant (k), and embryo sac (ES, arrowhead) of the wild type (j) and mutant (l). Note that there are no apparent differences in embryo sac development between the wild type and mutant plants. Bar = 30 μm.

microscope, anthers of the *cals5* mutants were found partially empty but containing shrunken and malformed pollen grains (Figure 4b,c,e,f). In comparison, the control anthers were filled with round and well-formed pollen grains (Figure 4a,d). To determine whether the malformed pollen grains were viable, Alexander solution (Alexander, 1969) was used to stain the pollen. Anthers of the wild-type plants contained purple-stained (viable) pollen grains (Figure 4a), whereas those of the *cals5* mutants were empty and pollen grains that remained were non-viable (green stain) (Figure 4b,c). Only a few purple pollen grains were occasionally observed in the mutant anthers (arrow in Figure 4c). Remnants of the degenerated pollen grains were visible in the anthers of the mutant plants (Figure 4e,f arrowhead). Although anthers of the *cals5* mutants were smaller than that of the wild type at an equivalent stage of development, they did not appear altered in their morphology. Transverse sections of the flower confirmed that these empty and smaller anthers still continued to develop and became partially dehiscent at the maturation stage (Figure 4e,f). Tapetal cells in the anthers of the *cals5* mutant were normal and apoptosed during pollen development as in the wild-type plants. The number of epidermal cells and endothecium cells around each pollen sac of the anther was about the same (18–20 cells), indicating that no sporophytic contribution occurs in the aborted pollen in the *cals5* mutant.

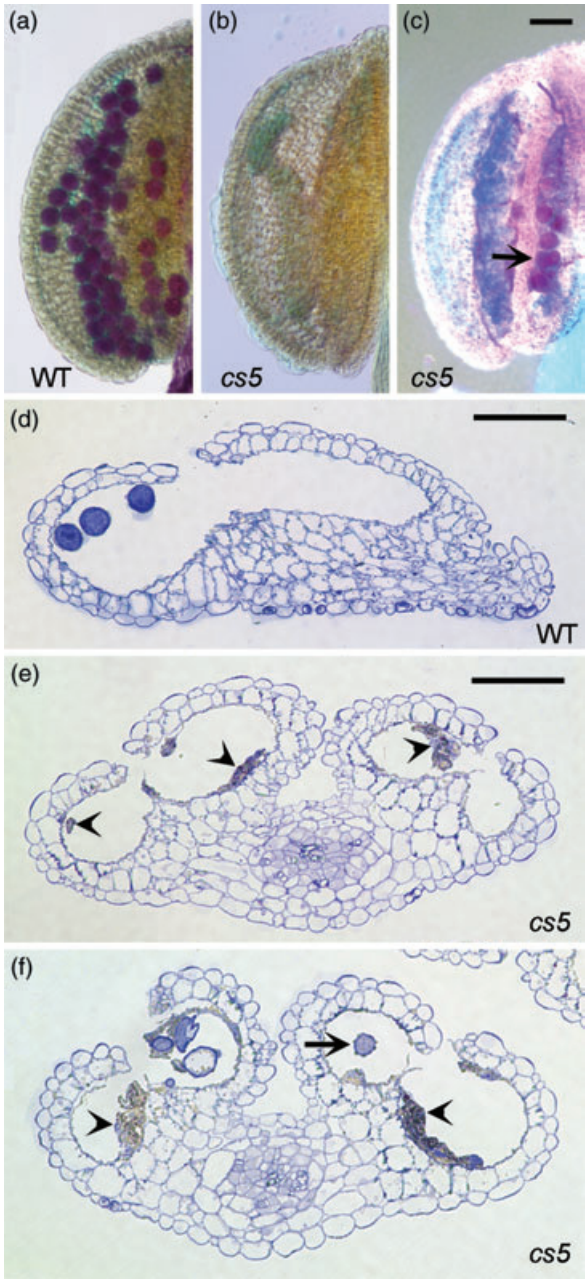


Figure 4. Arrest in microspore development in *cals5* mutants.
(a–c) Viability of pollen grains as assayed with Alexander staining. Anther of the wild-type plants is filled with viable, purple-stained pollen grains (purple; a), while that of the *cals5* mutants contains only non-viable pollen grains (green; b) or only a few viable pollen grains (arrow; c). Bar = 25 μm.
(d–f) Transverse sections of anthers. Wild-type anthers are dehiscent and contain round, well-developed pollen grains (d). Anthers of the mutants contain no pollen grains (e) or rarely a few grains (f). They are dehiscent at the mature stage and do not show an altered structure compared with that of the wild type. Note that degenerated remnants of microspores (arrowheads) are visible in the mutant anthers (e–f) but not in the wild type (d). Bars = 200 μm.

Tissue-specific expression of the CalS5 *gene*

To localize the expression of the *CalS5* gene at the cellular level, we cloned the promoter of this gene and used to drive the expression of β-glucuronidase (GUS) and GFP used as reporters (Figure 5). Arabidopsis plants expressing *CalS5:GUS* and *CalS5:GFP* constructs were produced using *Agrobacterium*-mediated transformation and used for histochemical analysis of the GUS activity and fluorescence detection of GFP in different plant tissues. As shown in Figure 5, *CalS5* was expressed in all cells of the root tip including root meristem, vasculature, pericycle, and cortex. The expression level in the maturation zone of the root was relatively weak (data not shown), which is in contrast to the expression patterns of other *CalS* genes including *CalS6*, *7*, and *8*, which are high in the vasculature of the elongation zone (Dong, 2004). In hypocotyl and stem, *CalS5:GUS* expression was primarily restricted to the vascular bundles, while little expression was found in the parenchyma and epidermal tissues. In cotyledons, leaves, and sepals, *CalS5:GUS* was expressed in the vascular bundles (Figure 5a–d). In flowers, *CalS5* was expressed at high levels in the anthers, particularly in pollen and embryos (Figure 5e,f). In contrast, four other *CalS* genes (*CalS9*, *10*, *11*, and *12*) were also expressed in pollen grains (Dong, 2004) but had much lower activity than *CalS5*. Apparently these genes cannot compensate for the loss of the *CalS5* gene in producing sufficient amount of callose to compensate for the phenotype. Transgenic plants expressing *CalS5:GFP* were also analyzed in order to follow the expression of *CalS5* gene in the living tissues. Expression of *CalS5:GFP* was detected throughout microsporogenesis, pollen development, and pollen tube growth (Figure 5h–s), which further supports the idea that CalS5 causes gametophytic male sterility.

Meiosis in the male gametophyte is normal in the cals5 *mutants*

We followed the progression of meiosis in *cals5* mutants in order to understand whether callose is involved in the process of meiosis in higher plants. The contents of anthers at various developmental stages were analyzed by staining for callose with aniline blue. Microsporocytes in both mutant and wild-type anthers appeared normal before the initiation of meiosis. However, sporocytes of the *cals5* mutants produced much less callose, when compared with that of the wild type (seen as bright blue fluorescence), but undergo apparently normal meiosis (Figure 6a–d). The four products of meiosis that were held together within the tetrads could be recognized in the *cals5* mutants (Figure 6e–h), although they appeared to clump together and the boundaries that separate the microspores were not as clear as those in the wild-type spores. Microspores of the mutant exhibited a faint aniline blue fluorescence when compared with the wild

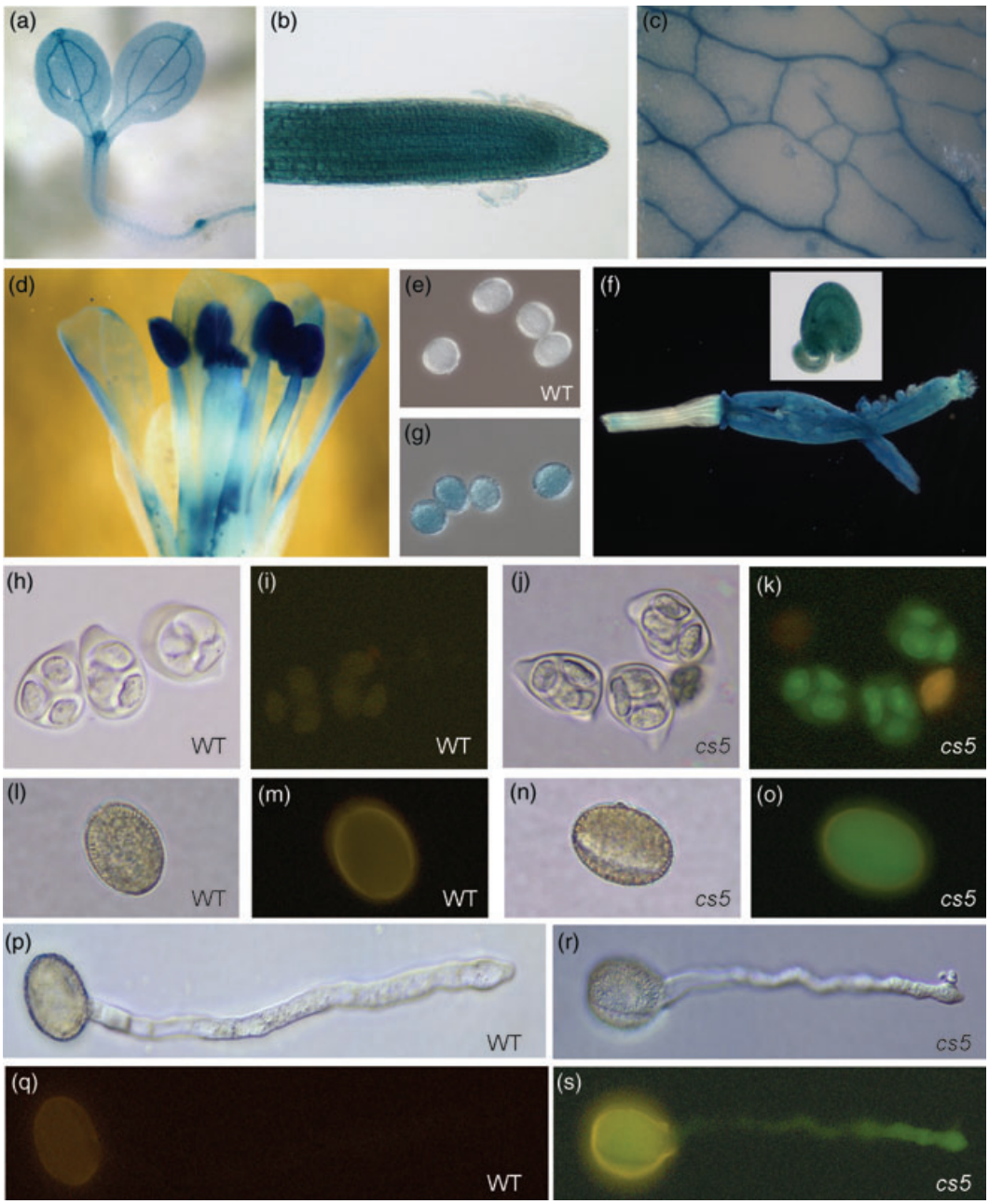


Figure 5. Tissue-specific expression of *CalS5:GUS* and *CalS5:GFP* in transgenic plants.
(a–g) GUS staining of plants expressing *CalS5:GUS*. Expression of *CalS5* was detected in the vascular tissues of cotyledons (a), leaf (c) and petals (d), in the shoot meristem (a), root tip (b), pollen grains (g), young silique (f) and developing seed (f, inset). (e) Pollen grains of wild-type plants.
(h–s) GFP fluorescence in living plants expressing *CalS5:GFP* (*cs5*). Expression of *CalS5* was detected in the tetrad (k), pollen grain (o) and growing pollen tube (s). Shown are bright field and fluorescence images of tetrad from wild type (h, i) and the transgenic line (j, k); pollen grain of wild type (l, m) and the transgenic line (n, o); and growing pollen tube of wild type (p, q) and the transgenic line (r, s).

type (Figure 6e–h). This may be due to the production of callose by CalS11 and 12 that are also expressed in pollen. Microspore release in the mutant plants was normal to that observed in the wild-type plants but a large number of the young pollen grains were found to be deformed or burst (Figure 6i–l). Mature pollen (both round and shrunken) from the mutant plants lacked callose on their surface (Figure 6m–p). The process of microsporogenesis thus appears to be unaffected in the *cals5* mutant. This was further confirmed by examining nuclear division using DAPI staining which showed that meiosis in male gametophytes of the *cals5* mutant was normal (Figure 6q–t). Callose deposition,

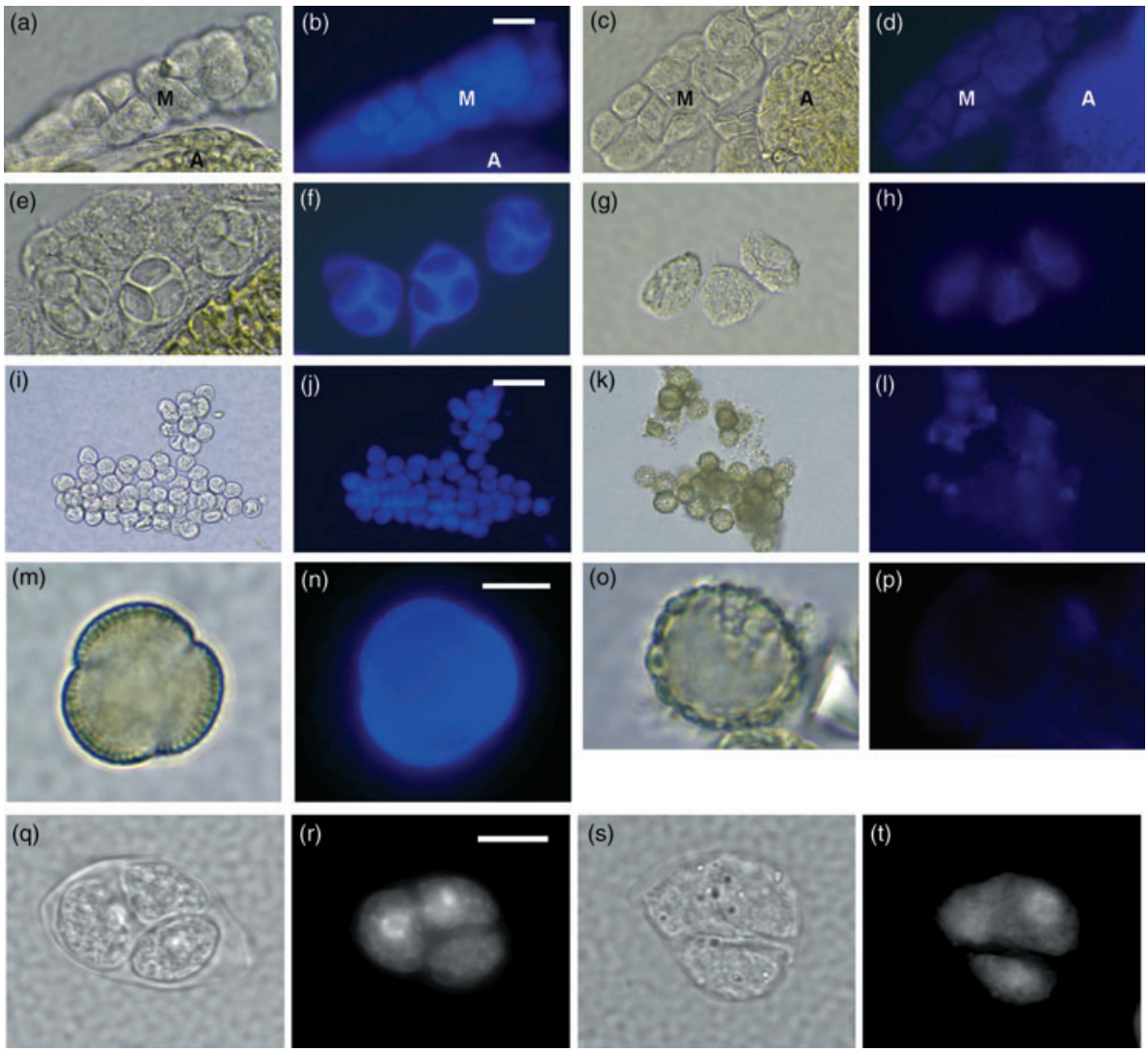


Figure 6. Callose deposition during microsporogenesis.
Reproductive tissues were fixed and stained with aniline blue. Bright field and UV fluorescence images were taken under a epifluorescence microscope. Note that callose deposition was nearly absent in meiocytes, tetrads, microspores, and pollen grains in the mutant plants.
(a–d) Meiocytes of wild type (a, b) and mutant (c, d). Bar = 10 μm for (a)–(h), 20 μm for (i)–(l).
(e–h) Tetrad of wild type (e, f) and mutant (g, h).
(i–l) Microspores of wild type (i, j) and mutant (k, l).
(m–r) Pollen grains of wild type (m, n), mutant pollen (o, p). Bar = 10 μm.
(q–t) Light and DAPI fluorescence micrographs of wild type (q, r), mutant tetrads (s, t). Bar = 5 μm.

however, appears to have a vital role in the process of microspore maturation, as the lack of callose affects pollen wall structure and viability of the pollen.

Defective exine formation in cals5 *mutants*

To understand the mechanism of pollen degeneration in *cals5* mutants, we compared the ultrastructures of meiocytes, tetrads, newly released microspores and the mature pollen grains of the mutant and wild-type plants using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In contrast to the reticulate pattern observed on the wild-type pollen, the pollen grains of the *cals5* mutant had a spotted pattern on the surface of the pollen (Figure 7i,j). At the meiocyte stage, there was no difference between the wild-type and the mutant plants (Figure 7a,b). However, at the tetrad stage, callose wall of the wild-type plants was thicker than that of the mutant, and plasma membrane of the mutants was wavy and irregular (Figure 7c,d). As the microspores released from the tetrad, the differences in pollen wall of the mutant and wild type became apparent. Tryphine granules were deposited on the

wall of the mutant pollen earlier than that in the wild type (Figure 7e,f). This might be due to the lack of callose which otherwise had to be removed in the wild type before tryphine deposition. Detailed observations using TEM suggested that the *cals5* mutant completely lacks the exine (baculum and tectum) structure (Figure 7g,h). Tryphine, derived mainly from the tapetum forms a pollen coat that filled the interstices of the exine in the wild-type pollen (Figure 7g), whereas it aggregated into numerous large agglutinations on the pollen surface in the *cals5* mutant (Figure 7h). In addition, numerous large agglutinations of sporopollenin-like material were found inside the mutant pollen that appeared to be endocytosed into the central vacuole, which also accumulated multivesicular bodies (Figure 7k). There was also a significant difference in the plasma membrane between the mutant and the wild-type pollen. The plasma membrane of the wild-type pollen was flat and the intine maintained the same thickness surrounding the plasma membrane (Figure 7g). In contrast, the plasma membrane of the *cals5* pollen was irregular and wavy. In addition, the intine surrounding the plasma membrane was irregular in thickness in mutant pollen (Figure 7h). Taken together, these data suggest that the callose wall of microspores may provide a structural basis for the formation of exine. Lack of the callose wall in *cals5* mutants may have two consequences: the exine cannot be formed and the intine becomes 'leaky,' having an active endocytosis on the plasma membrane. As a result, these microspores cease further development and eventually become deformed and degenerate. A dissection of the detailed mechanistic events leading to pollen death may eventually allow us to understand the precise role of callose in exine formation.

Discussion

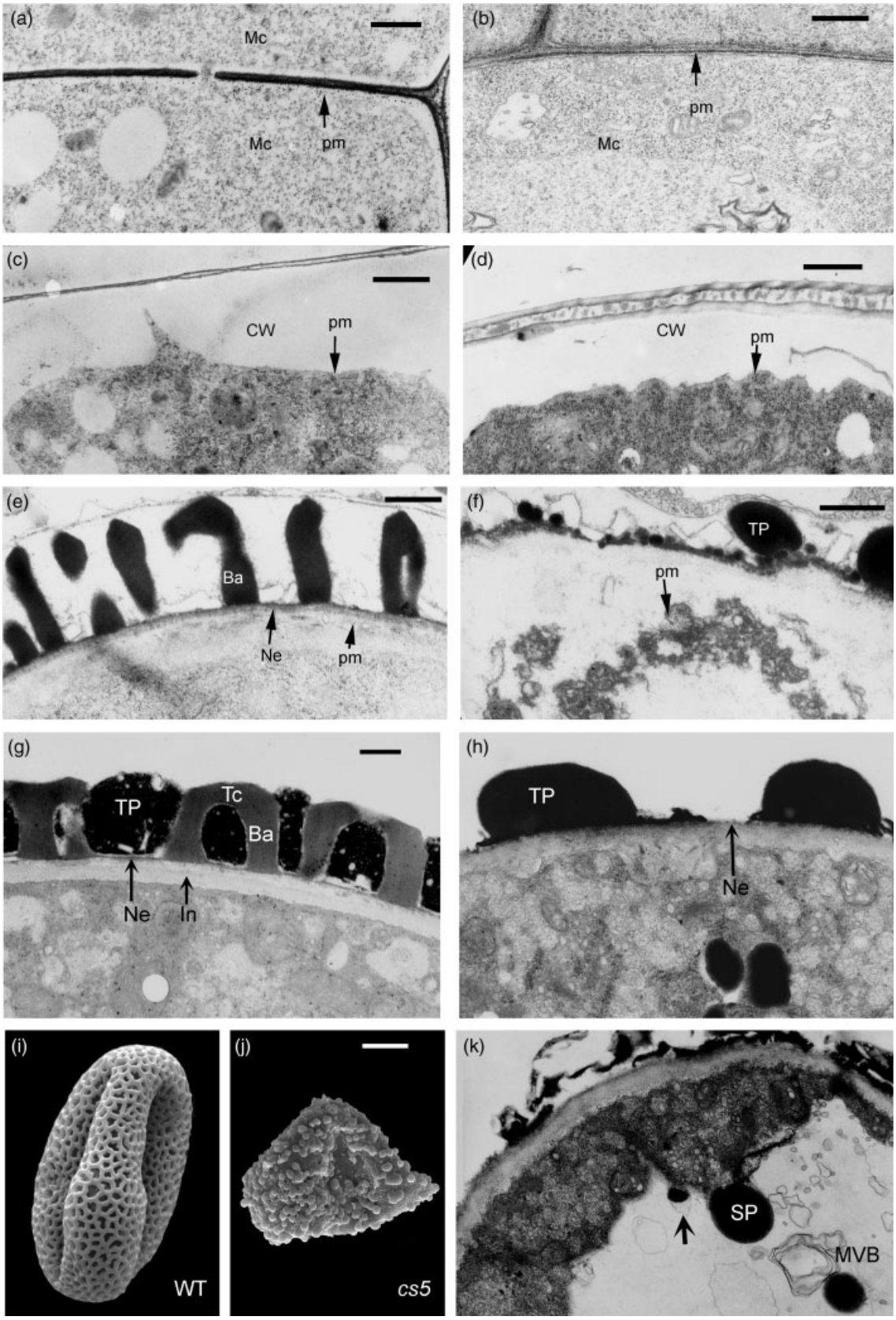
CalS5 *gene encodes a callose synthase required for microgametogenesis*

Two of the 12 *CalS* genes, *CalS1* and *CalS12*, have been characterized previously (Hong *et al.*, 2001a; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Østergaard *et al.*, 2002). In this report, the isolation and characterization of two insertional mutants of the *CalS5* gene was described and we demonstrate that callose synthesized by CalS5 is essential for the development of pollen wall resulting in fertile pollen in Arabidopsis. *CalS5* encodes a membrane protein of 1923 amino acid residues. Like other callose synthases in the CalS protein family, it contains multiple transmembrane domains that are clustered in two segments. The N-terminus (480 residues) and a large central hydrophilic loop (750 residues) of this protein are predicted to face the cytoplasm and may play a role in the interaction with other components of the callose synthase complex (Verma and Hong, 2001). The central loop contains the putative catalytic site which is characterized by the absence of the QXXRW motif that is highly conserved in the CesA superfamily of proteins (Saxena *et al.*, 1995; Verma and Hong, 2001). It appears that plant CalS may not directly bind UDP-glucose; instead, this function may be carried out by UDP-glucose transferase (UGT1) that contains a UDP-glucose binding signature and has been shown to tightly interact with the catalytic subunit of CalS1 (Hong *et al.*, 2001b). Results presented here clearly show that CalS5 is responsible for the synthesis of callose required for fertile pollen development in Arabidopsis. In the absence of this enzyme as shown by the knockout *cals5* mutants, callose is lacking on the cell wall of meiocytes, tetrads, microspores and pollen grains (Figure 6d,h,l,p,r) resulting in the development of sterile pollens.

CalS5 *is expressed highly in anthers and its role in microgametogenesis cannot be replaced by other* CalS *genes*

The expression of each of the 12 *CalS* genes in Arabidopsis is regulated differently in order to meet the demands for callose synthesis in different locations and in response to different physiological and developmental signals (Dong, 2004; Verma and Hong, 2001). CalS1 and CalS12 have been localized at the forming cell plate and to the site of pathogen infection, respectively (Hong *et al.*, 2001a; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). Consistent with their function in plants, the *CalS1* gene is expressed at high levels in the meristems where cell division and cytokinesis are active (Dong, 2004). The *CalS12* gene is induced during pathogen infection and knockout or knockdown mutations of this gene cause the depletion of callose around the infection site and render resistance to pathogens (Dong, 2004; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). The *CalS1* gene is also induced during pathogen infection (Dong, 2004), however, its role in disease resistance is not known. *CalS4* expression is confined to the primordia of branching axial buds (Dong, 2004) but the exact role of this gene in bud initiation and development is not known.

Although multiple *CalS* genes are expressed in the same cell type, e.g., *CalS1*, *2*, *3*, *5*, *6*, *7*, *8*, *9*, *10*, and *11* are expressed in the leaf (Dong, 2004), no apparent defects were observed in any tissue except the pollen of the *cals5* mutant plants. It is likely that the role of the CalS5 protein in other tissues of the *cals5* mutant partially overlaps the other *CalS* genes expressed in these tissues. In anthers, where *CalS5* is highly expressed, its role in microgametogenesis apparently cannot be replaced by other *CalS* genes such as *CalS9*, *10*, *11*, and *12* which are also co-expressed in the anthers but at low levels (Dong, 2004). This cell type-specific, high-level expression seems necessary for the formation of specialized cell walls in pollen. The possible contribution of *CalS9*, *10*, *11*, and *12* cannot be fully ruled out and can be assessed by double mutations.


(a)
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(d)
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TP
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(h)
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WT
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cs5
(k)
SP
MVB

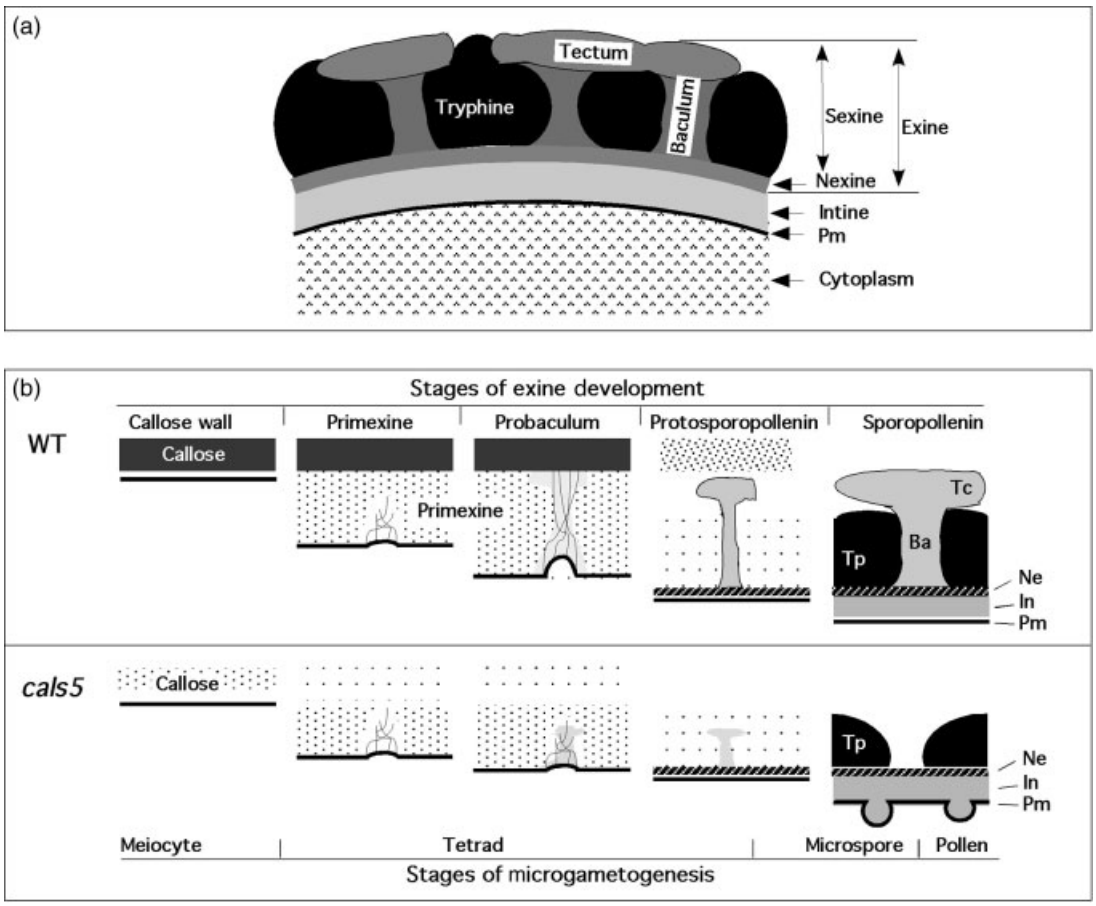

Figure 8. The proposed role of the callose wall in exine formation during microgametogenesis.
(a) Structure of a mature pollen grain wall. Pm, plasma membrane.
(b) Diagrammatic views of various stages of exine formation in the wild type (WT) and *cals5* mutant plants. Callose is deposited between the plasma membrane and the primary cell wall of the microsporocytes. It is eventually removed as the exine is laid down on the pollen wall. Upon completion of meiosis, the primexine is formed between the plasma membrane and the callose wall. The probaculum becomes evident at the locations where the plasma membrane protrudes into the expanded primexine layer. As the tetrad stage progresses, protosporopollenin is synthesized in the microspores and secreted only to the probaculae. As the primexine dissipates, the nexine is formed as a foot layer of the exine. During the late tetrad stage the callose wall begins to degrade by callase, a β-1,3-glucanase secreted by the tapetum cells. Following microspore release from the tetrads, protosporopollenin is rapidly converted to sporopollenin via cross-linking. Sporopollenin secreted from the tapetum cells plays an important role in the formation of the acetolysis-resistant exine pattern (baculae and tectum). Finally, the intine is formed and tapetally derived tryphine fills the interstices of the exine pattern. In the *cals5* mutant, the callose wall is not formed. Because the initiation of probaculae requires the callose wall for anchoring, probaculae, and thereafter baculae and the exine pattern cannot be formed in the *cals5* mutant. Coating with tryphine and the formation of nexine and intine layers are not affected by the lack of the callose wall. Tc, tectum. Ba, baculum. Tp, tryphine. Ne, nexine. In, intine. Pm, plasma membrane.

Callose is required for exine wall formation of pollen grains

In most plant species, the pollen grain wall is composed of an inner layer, intine, and an outer layer, exine. As outlined in Figure 8, the exine consists of a plain inner layer and an outer sculptured layer called nexine and sexine, respectively (Figure 8a). The sexine is built by rod-like structures called bacula which are subsequently covered by a roof (of different shapes) called tectum. In the final stage of wall development, additional proteins, phenolics, and fatty acid derivatives are deposited in the exine forming a layer called the tryphine (Osthoff and Wiermann, 1987; Owen and

Figure 7. Ultrastructures of meiocytes, tetrads, young microspores, and mature pollen grains.
(a, b) TEM of meiocytes (Mc) of wild type (a) and mutant (b). pm, plasma membrane. Bar = 0.5 μm.
(c, d) TEM of tetrads of wild type (c) and mutant (d). Note that callose wall (CW) of the wild type is thicker than that of the mutant. pm of mutant is waved irregularly. Bar = 0.5 μm.
(e, f) TEM of young microspores of wild type (e) and mutant (f). Note that the bacula (Ba) is formed upon nexine in wild type but not in mutant. Tryphine granules deposited on the callose wall of the mutant pollen at an early stage than in the wild-type pollen. Bar = 0.5 μm.
(g, h) TEM of mature pollen exine structure in wild type (g) and mutant (h) plants. Note the absence of Ba and tectum (Tc) in the mutant pollen. Bar = 0.5 μm.
(i, j) SEM of pollen grains of wild type (i) and mutant (j). Bar = 5 μm.
(k) The central vacuole in the mutant pollen contained numerous multivesicular bodies (MVB) and apparent endocytosed tryphine granules (SP) that were encapsulated by a membrane.

Makaroff 1995; Piffanelli *et al.*, 1998; Scott *et al.*, 1991). The exine pattern of pollen grains in *cals5* mutants was altered dramatically. The sexine, consisting of baculae and tectums, was missing whereas the tryphine that fills the interstices of sexine on wild-type pollen grains formed aggregates on the outer layer of the mutant pollen (Figures 7c,d and 8b). This exine-defective phenotype resembles that of transgenic plants expressing a callase (β-1,3-glucanase) in the anther locule (Worrall *et al.*, 1992) but is different from that of the *ms2*, *dex1*, *flp1*, and *nef1* mutants that have previously been characterized to be defective in exine formation. Sporopollenin failed to be deposited onto the microspore wall in *nef1* whereas it was randomly aggregated along the microspore wall in the *dex1* mutant (Ariizumi *et al.*, 2004; Paxson-Sowders *et al.*, 1997, 2001). The occasional few pollen grains produced by the *ms2* mutant have a very thin exine whereas the exine of the *flp1* mutant appears to be normal but is acetolysis sensitive (Aarts *et al.*, 1997; Ariizumi *et al.*, 2003).

The callose wall of tetrads acts as a mold which is filled by the primexine and on which sporopollenin is deposited leading to the formation of the final sculpted exine wall (Stanley and Linskens, 1974; Waterkeyn and Beinfait, 1970). In the *cals5* mutant, where callose is lacking in the cell wall of meiocytes, tetrads, and microspores (Figure 6), a proper exine wall fails to develop in the mature pollen (Figures 7 and 8). Without an exine sculpture, the sticky tryphine, which consists of various proteins, phenolics, and fatty acid derivatives, could not form a thin layer covering the pollen grains. Instead, it aggregated into electron-dense granules randomly distributed on the pollen wall of the *cals5* mutant (Figure 7). In the *dex1* mutant, in which callose deposition is normal, abnormal deposition of sporopollenin onto the plasma membrane also occurred (Paxson-Sowders *et al.*, 1997). In the *nef1* mutant that develops a normal callose wall, sporopollenin is not deposited onto the plasma membrane of microspores because of the lack of normal primexine; instead it aggregates and accumulates on the inner surface of the locule wall (Ariizumi *et al.*, 2004). In the *cals5* mutant, tryphine was only found on the pollen surface and in the remnant of the degenerated pollen wreckage, but not on the inner surface of the locule wall (Figure 4).

It appears that the timing of microsporogenesis is not affected by the change in the callose wall because the four microspores are produced and held together in a tetrad-like structure in the anthers of the *cals5* mutant (Figures 3 and 6). This suggests that material other than callose is capable of holding the microspores together in a tetrad. Alternatively, small amounts of callose synthesized in the *cals5* mutant by four other *CalS* genes (*CalS9*, *10*, *11*, and *12*) known to be expressed in this tissue (Dong, 2004), may be sufficient to hold the cells of the tetrad together. Single-gene mutations in these latter genes show no phenotype but double mutations may show some effect. A normal release of the microspores from the tetrad observed in the mutant anthers suggests that factors other than callase may also be required in this process (Sexton *et al.*, 1990; Steiglitz, 1977). Because cellulose is also part of the tetrad cell wall, cellulase may be required for the release of microspores.

Callose synthesis is important during megasporogenesis, microsporogenesis, and pollen tube growth (Stone and Clarke, 1992; Tucker *et al.*, 2001; Worrall *et al.*, 1992). *CalS2*, *3*, *6*, *7*, and *8* are co-expressed at the junction of filaments and anthers (Dong, 2004) suggesting that several CalS isoforms may be required for callose production in these tissues. It remains to be tested if different CalS isoforms interact with each other to form heteromeric complexes, or they form homomeric complexes that are co-localized (Verma and Hong, 2001). The fact that callose deposition affects exine formation suggests that this homopolymer (callose) interacts with other proteins to create a bridge with the plasma membrane where baculae are formed. As no marker for primexine has currently been identified, we do not know whether the primexine is actually formed in *cals5* mutants. The full protein composition of the pollen wall remains to be elucidated, including the role of various proteins in building the highly sculpted pollen wall structure necessary for the development of a fertile pollen.

Experimental procedures

Promoter-reporter constructs and plant transformation

The promoter region of the *CalS5* gene was amplified by PCR using genomic DNA from *Arabidopsis thaliana*, ecotype Columbia. The forward primer for *CalS5* gene was designed to start from the end of the immediate upstream gene. The reverse primers were designed to amplify the coding regions of the *CalS5* gene for the first 20 amino acid residues from the N-terminus. PCR-amplified fragments were cloned in pCR2.1 (Invitrogen, Carlsbad, CA, USA), verified by DNA sequencing and subcloned into pBI101.2 vector (DB Biosciences, Palo Alto, CA, USA) as a translational fusion in frame with the GUS coding region. The construct was electroporated into *Agrobacterium tumefaciens* strain GV3101 and used to transform Arabidopsis plants via flower infiltration. Kanamycin (50 μg ml^{-1})-resistant seedlings were selected, transferred to soil, and grown in a growth chamber. The promoter fragment was cloned in pENTR1A and then subcloned into pBGWFS7 as a translational fusion in-frame with GFP by LR recombination reaction (Invitrogen). The construct was electroporated into *A. tumefaciens* strain ABI and used to transform Arabidopsis plants via flower infiltration. BASTA (50 μg ml^{-1}; Sigma-Aldrich, St Louis, MO, USA) in water was sprayed on 6-day-old seedlings after germination and was applied three times every other 3 days. BASTA-resistant seedlings were selected, transferred to pots, and grown in a growth chamber.

GUS staining and GFP detection

Histochemical staining for detecting GUS expression was performed as described (Jefferson *et al.*, 1987). After staining, chlorophyll was extracted from photosynthetic tissues with 70% ethanol. Fifteen transgenic lines expressing either *CalS5:GUS* or *CalS5:GFP* construct were analyzed and one representative was selected for

further experiments. Localization of GFP was performed using an epifluoresence microscope.

RNA extraction and RT-PCR

Total RNA was isolated from flowers using the Trizol kit (Invitrogen). Reverse transcription of RNA (50 ng) was carried out using SuperScript II (Invitrogen) and PCR was performed for 25 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min). Primers CalS5F, 5′-TTGCAGCGAAAGG-AGATAGTTCTCTT-3′, CalS5R, 5′-TTATTTCTGCTTCTTACCACC-3′ were designed to amplify PCR products of approximately 1200 bp. RT-PCR of actin-2 served as an internal control.

Genomic DNA extraction and isolation of a T-DNA insertion line

Seedlings of T-DNA insertional lines Salk_009234 for *CalS5-1* and Salk_026354 for *CalS5-2* were grown individually. One fully expanded leaf of a 4-week-old plant was used to extract genomic DNA. Two *CalS* gene-specific primers (CS5-1-LP: 5′-TGCAACTCAGGATCC-ATTTTCTTG-3′ and CS5-1-RP: 5′-CCAGAAAAACTACCTACCTCTC-CCAAACG-3′ for *CalS5-1* or CS5-2-LP: 5′-TGCTTCTGTGGTGGTC-ACAGG-3′ and CS5-2-RP: 5′-GCATACCAAATTTGAGTGT-CCAT-3′) and one T-DNA primer (LBb1: 5′-GCGTGGACCGCTTGCTGCAACT-3′) were used in PCR using genomic DNA as template (Siebert *et al.*, 1995). Genomic DNA from wild-type plants produced a PCR product of approximate 907 and 971 bp, whereas homozygous plants produced a 600-bp PCR band for *CalS5-1* and 650 bp for *CalS5-2*. Both PCR bands were present using genomic DNA from heterozygous plants. Seeds from homozygous plants, designated as *cals5-1* and *cals5-2*, were collected and used for further experiments.

DAPI staining, callose staining, and pollen viability assay

Microspores were stained with DAPI for 10 min and observed using light microscopy and epifluorescent microscopy (Park *et al.*, 1998). For callose staining, flowers were fixed in ethanol/acetic acid (3:1, v/v) and stained with 0.01% aniline blue (Hong *et al.*, 2001a). The tissue was viewed in a fluorescent microscope using a UV filter. For pollen viability assay, anthers were stained in Alexander solution and observed by light microscopy (Alexander, 1969). DIC microscopy was used to observe anthers or ovules that had been cleared using Herr's solution (phenol:chloral hydrate:85% lactic acid:-xylene:oil of clove, 1:1:1:0.5:1) (Stevens *et al.*, 2004).

Light microscopy

Anthers were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0 and were post-fixed in 2% osmium in the same buffer. An ethanol dehydration series was performed and the samples were embedded in Spurr resin. Transverse sections of 1.5 μm were cut using an Ultratome III ultramicrotome (LKB, Sweden) stained with 0.5% toluidine blue, and observed with a light microscope.

Electron microscopy

For SEM, fresh pollen grains were coated with 8 nm gold, and observed on a JSM-8404 microscope (Jeol, Japan). For TEM, anthers were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0 and were post-fixed in 2% osmium in the same buffer. Following ethanol series dehydration, samples were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate. Micrographs were taken using a Phillips CM12 transmission electron microscope (Philips Electronic Instruments Co., Mahwah, NJ, USA) at 60 kV.

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