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# Pollen Exine Pattern Formation is Dependent on Three Major Developmental Processes in *Arabidopsis thaliana*

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## ABSTRACT

Classical ultrastructural studies have revealed that the architectural structure of the pollen wall is composed of a series of concentric outer layers, although its shape, size and morphology are highly diverged among plant species. These layers are known as the exine, which is formed around the microspore during microsporogenesis. Detailed morphological studies have demonstrated that the exine consists of the simple inner nexine layer and the outer sexine portion, which determines the sculptured exine structure. Biochemical studies have shown that a constituent of the exine precursor, sporopollenin, potentially contains polymers of fatty acid, phenylpropanoids and phenolics derivatives. On the other hand, genetic and molecular studies employing *Arabidopsis* mutants defective in exine formation have provided new knowledge not only on the critical processes for this pattern formation, but also on the genes involved in the process. Characterization of these mutants has shown that they can be generally classified into three types from a morphological viewpoint: mutants defective in sporopollenin synthesis, primexine formation or callose wall formation. The genetic approach has demonstrated that *Arabidopsis* mutants lacking any of these three processes show failure in exine pattern formation. In other words, these three processes play critical roles in exine pattern formation in *Arabidopsis*. Here we review the mutants and genes related to exine pattern formation in *Arabidopsis*.

**Keywords:** exine formation, sporopollenin, primexine, callose wall, *Arabidopsis*
**Abbreviations:** **CV**, coated vesicle; **FAA** formalin/alcohol/acetic acid, **LTP12**; lipid transfer protein12; **PCD**, programmed cell death; **PDH**, pyruvate dehydrogenase; **PR**, pathogenesis-related; **TCA**, tricarboxylic acid; **VLCFA**, very long chain fatty acid; **WT**, wild-type
**Arabidopsis mutants:** ***apt1***, *adenine phosphoribosyltransferase1*; ***cals5***/***lap1***, callose *synthase5*/*less adherent pollen1*; ***copt1***, *ctr-related copper transporter*; ***dex1***, *defective in exine formation 1*; ***kom***, *kompeito*; ***ms1***/***hkm***, *male sterile1*/*hackly microspore*; ***ms2***, *male sterility2*; ***ms9***, *male-sterile9*; ***ms12***, *male-sterile12*; ***nef1***, *no exine formation1*; ***wax2***/***yre***/***flp1***, *wax2*/*yore-yore*/*faceless pollen-1*

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Invited Review

## INTRODUCTION

### Exine structure

The Angiosperm pollen grain is surrounded by a pollen wall structure, which is believed to play a role in protecting pollen from threats such as bacterial and fungal attacks and severe environmental conditions. The pollen wall consists of three layers, the exine (approximately 1.0 μm in thickness), intine (approximately 0.2 μm in thickness) and tryphine (or pollen coat, approximately 0.95 μm in thickness). The exine is the outermost layer of the pollen wall that is responsible for sculpturing pollen structure. Reticulate exine patterning, in particular, can be found in Brassicaceae species (**Fig. 1A**). Although the shape, size and morphology of pollen grains vary among species in angiosperms, their exine structure is basically identical. The exine consists of two layers, the outer sexine and the inner nexine (**Fig. 1B**). The sexine contains an outermost edge, the tectum, and the radially directed rods, the bacula. These two portions sculpture a species-specific structure of pollen grains, whereas the nexine is a simple layer that is laid down on the intine layer. The intine is a simple layer consisting of cellulose and pectin, while the tryphine is a layer which coats pollen grains. The tryphine includes fatty acid derivatives such as esters and lipidic volatile compounds and various proteins (Wolters-Arts *et al*. 1998; Mayfield and Preuss 2000; Mayfield *et al*. 2001). The tryphine, however, is not important for pollen adhesion to the stigma cells since pollen from tryphine-lacking *Arabidopsis cer6-2* mutant can adhere to the stigma cells (Zinkl *et al*. 1999). It is the pollen exine that is important for adhering to the female stigma cell that makes it possible to transmit pollen gametes. This adhesion takes place within seconds after pollination, prior to pollen hydration. The adhesion molecules are probably lipophilic molecules, and are most likely to reside within the exine (Zinkl *et al*. 1999). The exine determines a species-specificity of adhesion, which enables flowering plants to bind pollen grains only from appropriate species onto the stigma cells.

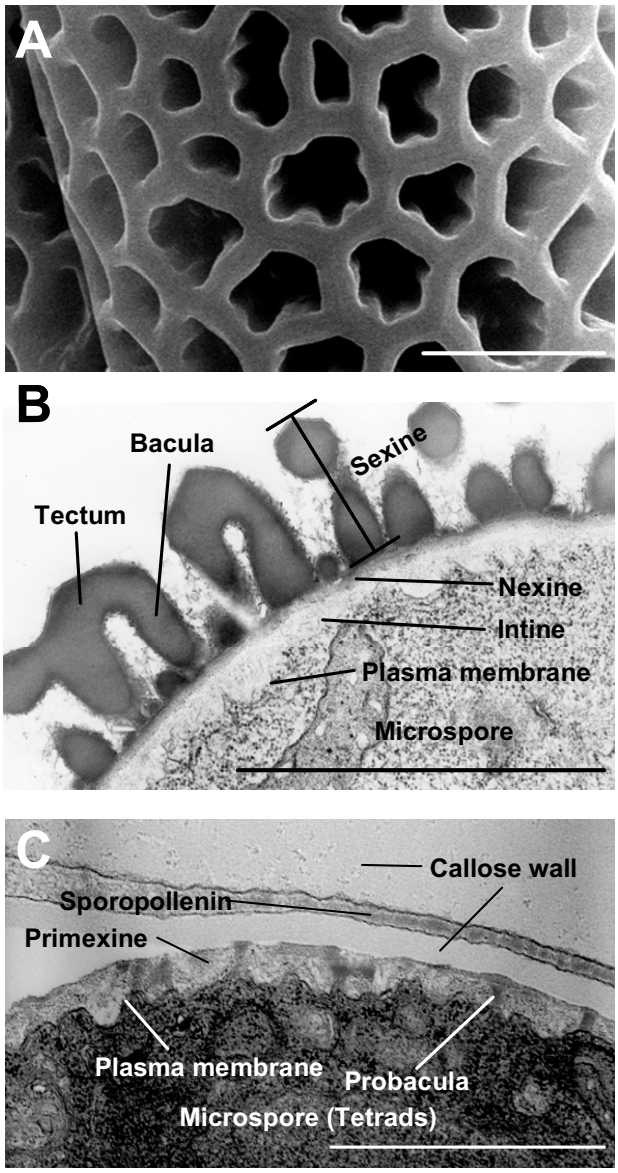


**Fig. 1 Micrographs of pollen wall and exine structure in *Arabidopsis*.** (**A**) Scanning electron micrograph of mature pollen grain. Reticulate exine patterning is evident in *Arabidopsis*. (**B**) Transmission electron micrograph of a cross-section of exine structure in mature pollen grain. Roof structure, the tectum, and rod structure, the bacula, are evident. (**C**) Transmission electron micrograph of cross-section of microspores at the tetrad stage. Primexine formation is evident between the callose wall and undulation plasma membrane. Sporopollenin deposits are visible outside of the callose wall. Bars = 2.0 μm.

### Sporopollenin – exine constituent

Biochemical analysis for determining the constituents of exine has been widely carried out, and has clarified a possible component of exine, which is termed sporopollenin. Sporopollenin is used as a generic term for the exine constituent. Several lines of biochemical evidence clearly indicate that sporopollenin consists of phenylpropanoids, phenolics and fatty acid derivatives. Osthoff and Wiermann (1987) have purified exine from pine pollen (*Pinus mugo* Turra), and proven that phenolic and aromatic compounds are located on and/or in the structures of the exine. Wilmesmeier and Wiermann (1995) have shown that sporopollenin biosynthesis of exine in *Zea mays* is influenced by the use of thiocarbamate herbicides which reduce the synthesis of very long chain (>C18) fatty acids (VLCFA), indicating that fatty acid elongase systems of lipid metabolism are involved in sporopollenin biosynthesis. The pollen exine develops as a result of sequential polymerization of sporopollenin. It is known that once the degree of sporopollenin polymerization proceeds, the exine tends to acquire a physical strength and extreme resistance to non-oxidative chemical and biological degradation (reviewed by Scott 1994; Ahlers *et al*. 1999; Meuter-Gerhards *et al*. 1999).

Several reports have suggested that sporopollenin also contains carotenoids, although an inhibitory experiment using norflurazaon that blocks carotenoid biosynthesis failed to prevent sporopollenin synthesis in *Cucurbita pepo* (Prahl *et al*. 1985; reviewed by Scott 1994).

### Callose wall formation

A microsporocyte is surrounded by a special wall, the callose wall, which consists of β-1,3-glucan, and microsporocyte meiosis takes place within the callose wall (**Figs. 1C, 2A**). Callose is a temporary cell wall between the plasma membrane and primary cell wall, and this callose wall synthesis begins from the microsporocyte meiosis stage (Worrall *et al*. 1992). It is suggested that the callose wall may function as a protector of the developing microspores from the influence of surrounding tissues, and/or as a physical barrier of developing microspores to prevent them from premature degradation (Knox and Heslop-Harrison 1970). The callose wall persists in the locule until the tetrad stage, and the wall is known to be degraded by β-1,3-glucanase (callase) which is secreted from the tapetum. The tapetum is one of the four anther walls, and it surrounds the anther locule (**Figs. 2, 3**). It is known that the tapetum secretes not only β-1,3-glucanase, but also lipidic molecules and nutriaents important for microspore development (reviewed by Piffanelli *et al*. 1998).

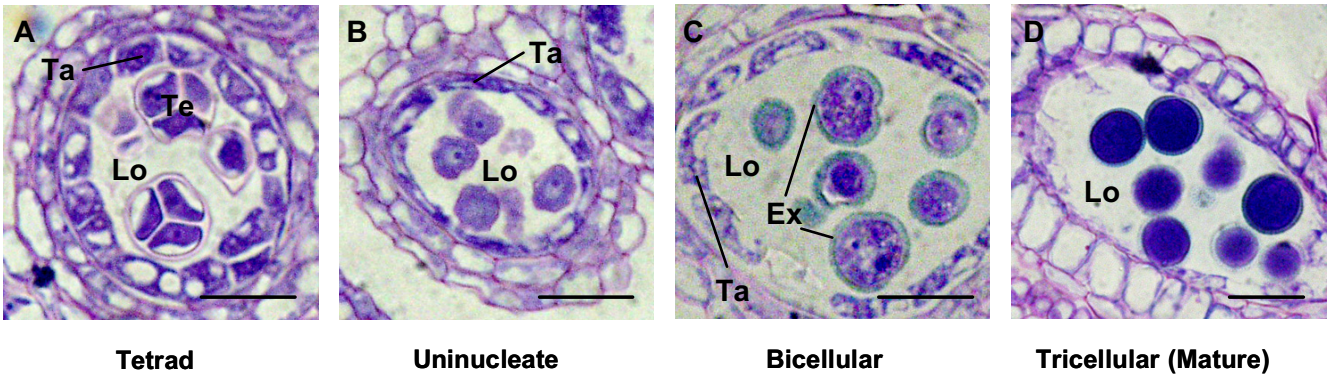


**Fig. 2 Cross sections of developing anthers.** (**A**) Light microscopy photograph of cross-sections of anther at the tetrad stage. Tetrad microspores are covered by callose wall. (**B**) Cross-section of anther at the uninucleate microspore stage. Microspores are released after dissolution of callose wall. (**C**) Cross-section of anther at the bicellular pollen stage. Thick exine which surrounds microspore is visible. (**D**) Cross-section of anther at the tricellular pollen stage (mature stage). Tapetum disappears as a result of its degradation. Ta, tapetum; Te, tetrad; Lo, locule; Ex, exine. Bars = 20 μm.

## Primexine formation and sporopollenin deposition

Primexine is a cellulosic matrix that functions as a scaffold of sporopollenin deposition (**Fig. 1C**). Primexine formation first takes places between the microspore and the callose wall at the later tetrad stage (Rhee and Somerville 1998). The initial process of sporopollenin polymerization takes place in the primexine as a probacula formation. The probacula begins to appear at the outside of the primexine (callose wall side), and the probacula is not in direct contact with the microspore plasma membrane (**Fig. 4**; Paxson-Sowders *et al*. 2001). At a later stage, the probacula makes contact with the membrane when the probacula becomes clearly visible in the primexine. When microspores are released in the locule after the degradation of callose wall by β-1,3-glucanase, the fundamental exine structure is already established, although the size of the exine remains relatively small at this stage. After the microspore release, the bacula and tectum continue to increase in size by sequential sporopollenin polymerization, and the sculpturing structure is almost completed by the time microspores undergo mitotic division to produce bicellular pollen (Owen and Makaroff 1995; Paxson-Sowders *et al*. 1997; Ariizumi *et al*. 2004).

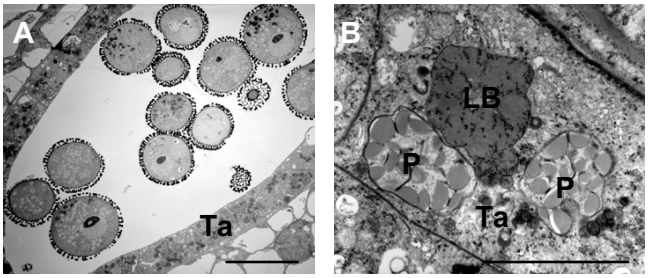


**Fig. 3 Lipid accumulation in tapetum.** (**A**) Transmission electron micrograph of cross-section of anther locule. Tapetum surrounds anther locule. (**B**) Lipid accumulation both in plastids and lipid bodies is evident in the tapetum. Lipid accumulation can be observed as high degree of electron density. LB, lipid body; P, plastid; Ta, tapetum. (**A**) Bar = 20 μm (**B**) Bar = 2.0 μm.

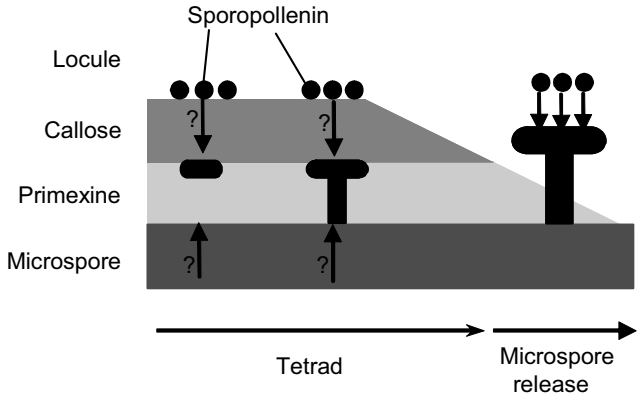


**Fig. 4 Schematic model of exine formation.** At the late tetrad stage, the primexine appears between the microspore plasma membrane and callose wall. Tapetal-derived sporopollenin aggregates on the surface of callose wall. Sporopollenin deposition starts to be visible at the locule side of the primexine. As stage proceeds, probacula formation becomes complete as a result of sporopollenin polymerization that causes rod structure contract with the plasma membrane. After the callose wall dissolution, tapetal-derived sporopollenin polymerizes further, and fundamental exine structure is evident at this stage. Further polymerization produces thick exine structure until bicellular or tricellular pollen stage.

## Tapetum and tryphine

The tapetum is a tissue in which lipid metabolism and accumulation are actively occurring and a number of genes associated with its metabolism are expressed during exine formation (**Figs. 2, 3A**; reviewed by Piffanelli *et al*. 1998). There are two major organelles in the tapetum, lipid bodies and plastids, and the lipid components are highly accumulated in these organelles (**Fig. 3B**). These morphological characteristics have been well examined in several plant species, and the biochemical characteristics have also been determined by analyzing lipid and fatty acid composition of these organelles (Wu *et al*. 1997; Hernandez-Pinzon *et al*. 1999). The tapetum eventually degenerates later in pollen development after the pollen attains a tricellular nucleus (**Fig. 2D**), and these remnants of lipid-accumulated organelles are finally deposited into the exine cavities as tryphine. It has been shown that the onset of pollen hydration and rapid water transfer are mediated by tryphine (Wolters-Arts *et al*. 1998; Mayfield and Preuss 2000).

## Recent progress in genetic studies

Previous cytological, physiological and biochemical studies for exine pattern formation have been well reviewed by several groups (reviewed by Scott *et al*. 1991; Scott 1994; Piffanelli *et al*. 1998; Scott *et al*. 2004; Boavida *et al*. 2005). On the other hand, recent genetic screening of exine-defective mutants has resulted in great progress to elucidate the developmental and molecular mechanism in exine pattern formation (**Table 1**). This recent progress has revealed that the exine pattern formation involves at least three proper developmental processes, sporopollenin synthesis, primexine formation and callose wall formation. It appears that these three factors are essential steps for exine pattern formation, and a lack of any processes results in failure of proper formation. Here we describe the mutants and genes related to sporopollenin synthesis, primexine formation and callose wall formation in *Arabidopsis*.

**Table 1** Predicted function of exine-associated genes and their mutant phenotypes.

| Name | Gene code | Predicted gene function | Sporopollenin | Primexine | Callose wall | Reference |
|---|---|---|---|---|---|---|
| MS2 | At3g11980 | Fatty acyl reductase | Defective | N.D. | N.D. | Aarts *et al*. 1997 |
| WAX2/YRE/FLP1 | At5g57800 | Fatty acyl dehydrogenase; Wax biosynthesis or transport? | Defective | N.D. | Normal | Chen *et al*. 2002; Kurata *et al*. 2002; Ariizumi *et al*. 2003 |
| DEX1 | At3g09090 | Unknown; Precursor of primexine? Rough ER? | Normal | Defective | Normal | Paxson-Sowders *et al*. 1997, 2001 |
| NEF1 | At5g13390 | Unknown; Maintain integrity of plastid membrane? Fatty acid biosynthesis or transport? | Defective | Defective | Normal | Ariizumi *et al*. 2004 |
| MS1/HKM | At5g22260 | PHD type transcription factor | Defective | Defective | Normal | Wilson *et al*. 2001; Ito and Shinozaki 2002; Ariizumi *et al*. 2005; Vizcay-Barrene and Wilson 2006 |
| APT1 | At1g27450 | Adenine phosphoribosyltransferase1 | Normal | Defective | Normal | Zhang *et al*. 2002 |
| CALS5/LAP1 | At2g13675 | Callose synthesis | Normal? | N.D. | Defective | Dong *et al*. 2005; Nishikawa *et al*. 2005 |
| KOM | Unknown | Callose synthesis? | N.D. | N.D. | Defective | Kanaoka *et al*. 2003 |
| MS9 | Unknown | Unknown | N.D. | N.D. | N.D. | Tylar *et al*. 1998 |
| MS12 | Unknown | Unknown | N.D. | N.D. | N.D. | Tylar *et al*. 1998 |
| COPT1 | At5g59030 | Copper transporter | N.D. | N.D. | N.D. | Sancenon *et al*. 2004 |

## SPOROPOLLENIN SYNTEHSIS AND DEPOSITION

### *male sterility2* (*ms2*) mutant

The *Arabidopsis male sterility2* (*ms2*) mutant produces pollen without the exine layer (Aarts *et al*. 1997). The *ms2* mutant produces irregular microspores after the tetrad stage, and almost every pollen grain collapses at anthesis, while vegetative defects are not observed. Large vacuoles are formed in the tapetum in the *ms2* mutant in comparison to the WT (wild-type) at the tetrad stage, when microsporocytes start meiosis to form a tetrad. Abnormal vacuolation further proceeds in the tapetum after the tetrad stage, and the vacuolated tapetum collapses the developing microspores. The occasional pollen grains produced by the *ms2* mutant show no sign of exine formation. Instead, an unknown, but a relatively electron-dense thin layer covers the pollen grains. These pollen grains are shown to be degraded after acetolysis treatment, while WT pollen grains are intact after the treatment, indicating that the electron-dense layer does not function as the exine itself. The MS2 protein shows highest sequence similarly to a protein encoding a jojoba (*Simmondsia chinensis*) fatty acyl reducetase involved in the formation of seed wax esters by reducing wax fatty acids to the corresponding wax alcohols (Shockey *et al*. 1995). The MS2 protein also shows additional sequence similarity to a number of other reductase proteins. Based on an *in situ* RNA hybridization experiment, *MS2* mRNA expression has been shown to be found only in the tapetum at the moment young microspores are released from the tetrads. Thus these results suggest that sporopollenin synthesis occurs in the tapetum, and that MS2 might reduce VLCFA to fatty alcohol at that time, and thus, this reaction might be one of the steps in sporopollenin synthesis (Aarts *et al*. 1997). It is important to determine whether MS2 protein has actual biochemical activity to reduce long chain fatty acid to fatty alcohols. Wang *et al*. (2002) have identified three putative orthologs of the *MS2* gene in wheat, which are designated *TRITICUM AESTIVUM ANTHER1a-1c* (*TAA1a-1c*). The authors demonstrated that one of the orthologs, TAA1a, has the kind of biochemical activity to produce fatty alcohols, suggesting that MS2 might act as a real fatty acyl reductase.

### *faceless pollen-1* (*flp1*) mutant

The *Arabidopsis faceless pollen-1* (*flp1*) mutant shows male sterility under normal conditions, although its fertility is restored under high humidity conditions (Ariizumi *et al.* 2003). In the *flp1* mutant, many parts of the exine are broken apart after the treatment of FAA (formalin/alcohol/acetic acid), although its microspores and their exine are visually normal without treatment. This implies that the extent of sporopollenin polymerization in the exine may be low in the mutant. The *flp1* mutant also shows defects in wax crystal deposition on the stems and siliques, and it has smaller lipid droplets in the tryphine compared to that of the WT. The *FLP1* gene is identical to the *WAX2* and *YORE-YORE* (*YRE*) genes, which are predicted to have six transmembrane domains and to localize at the plasma membrane (Chen *et al*. 2003; Kurata *et al*. 2003). The *WAX2*/*YRE*/*FLP1* encodes a 632 amino acid protein with a sequence similarity to the sterol desaturase family proteins and the EPI23 protein from *Senecio odora* which is suggested to be involved in epicuticular wax biosynthesis as a receptor transporting intermediate or the end-product of wax biosynthesis (Hansen *et al*. 1997). The WAX2/YRE/FLP1 also contains three histidine-rich motifs ($HX_3H$, $HX_2HH$ and $HX_2HH$, in which X stands for any amino acid), which is believed to be a catalytic domain in several types of enzymatic proteins such as fatty acyl desaturases and xylene monooxygenases (Shanklin *et al*. 1994). Lipid analysis has demonstrated that the amount of several types of VLCFA groups is decreased in the mutant compared to the WT (Chen *et al*. 2003; Kurata *et al*. 2003). These results suggest that the mutant is defective in sporopollenin synthesis and/or transporting of VLCFA.

The importance of VLCFA in exine formation is also observed in monocot rice plants. Jung *et al*. (2006) isolated a rice male sterile *wax-deficient anther1* (*wda1*) mutant, which lacks cuticular waxes both in vegetative and reproductive tissues. The *wda1* mutant produces microspores without the exine. Instead, a sporopollenin aggregation is deposited on the microspore surface at the pollen mitosis stage. Biochemical analysis to determine the wax composition in rice anthers demonstrated that the major wax composition comprised alkenes and alkanes with very long chains, and that these amounts of wax are considerably reduced in the *wda1* mutant compared to the WT. The *WDA1* gene encodes a membrane integral protein with five transmembrane proteins and with one histidine-rich motif. The WDA1 protein shows the highest sequence similarity to CER1 (ECERIFERUM1) among the *Arabidopsis* genome (Aarts *et al*. 1995), therefore, WDA might be the CER1 ortholog in rice, or it may be involved in the general processes of VLCFA biosynthesis (Jung *et al*. 2006).

### Down-regulation of pyruvate dehydrogenase (PDH)

Similar to the *ms2* mutant, an exine-less phenotype with no deposition of sporopollenin has also been reported in transgenic tobacco plants with the antisense gene of mitochondrial pyruvate dehydrogenase (PDH) under the control of the tapetum-specific *TA29* promoter (Yui *et al*. 2003). PDH is essential for the operation of the tricarboxylic acid (TCA)

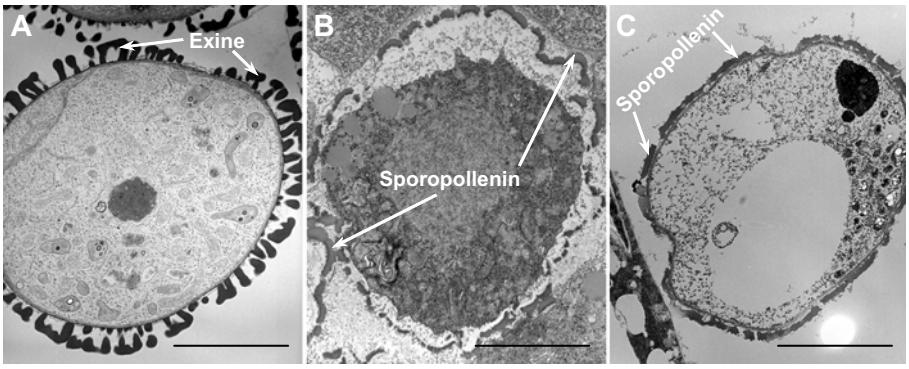


**Fig. 5 Exine-defective microspores in *nef1* and *hkm* mutants.** (**A**) Transmission electron micrograph of cross-section of WT at the uninucleate microspore stage. Sculpturing exine structure is evident at this stage. (**B**) Micrograph of *nef1* mutant at the uninucleate microspore stage. Sporopollenin never anchors to the microspore plasma membrane. Instead, sporopollenin aggregates are visible around the microspore. (**C**) Micrograph of *hkm* mutant at the uninucleate microspore stage. Sporopollenin deposits on the microspore plasma membrane. However, aberrant exine structure is visible. Bars = 5.0 μm.

cycle. The transgenic plants have poorly developed plastids and lipid bodies in the tapetum, suggesting that fatty acid synthesis is disturbed by the absence of the mitochondrial TCA cycle. As acetyl-CoA is formed directly from pyruvate via a pyruvate dehydrogenase complex in mitochondria, and the released acetyl-CoA from mitochondria can be used as a substrate for *de novo* fatty acid synthesis in plastids (Fischer and Weber 2002), it is suggested that the absence of PDH in the mitochondria fails to release fatty acid resources for exine formation (Yui *et al*. 2003). These genetic examples support the biochemical evidence that the sporopollenin constituent includes fatty acid derivatives.

## PRIMEXINE IS ESSENTIAL FOR SPOROPOLLENIN DEPOSITION

### *defective in exine formation 1* (*dex1*) mutant

A male sterile *defective in exine formation 1* (*dex1*) mutant of *Arabidopsis* produces irregular microspores after the tetrad stage similar to that of the *ms2* mutant (Paxson-Sowders *et al*. 1997, 2001), while a vegetative defect is not observed in the *dex1* mutant. Microspores and their plasma membranes develop in a similar manner to WT until the early tetrad stage. Later in development, however, the plasma membrane structure becomes less shallow than that in the WT microspore. The primexine is formed in the *dex1* mutant, but its deposition is delayed, and is less evident compared to that of the WT. Also, probacula formation is not observed in the *dex1* mutant. The sporopollenin seems to be synthesized normally, but is randomly deposited into the primexine. The sporopollenin, however, never anchors to the plasma membrane, but instead produces sporopollenin aggregation around the microspore, and the immature microspores finally degrade. *DEX1* encodes a 896 amino acid protein, and is predicted to have a transmembrane domain and to localize at the plasma membrane. DEX1 protein shows very limited sequence similarity to a hemolysin-like protein from *Vibrio cholerae*. An approximately 200 amino acid segment of DEX1 (439-643 amino acid residues) also shows limited sequence similarity to a calcium-binging domain of animal α-integrin. There are two sets of putative calcium-binding domains in this region, suggesting that DEX1 may be a calcium-binding protein. These results suggest that DEX1 is a component of the primexine matrix or rough endoplasmic reticulum (ER), and is involved in the assembly of primexine precursors to the plasma membrane (Paxson-Sowders *et al*. 1997, 2001).

### *no exine formation1* (*nef1*) mutant

The defect in primexine formation is also observed in the male sterile *no exine formation1* (*nef1*) mutant of *Arabidopsis* (Ariizumi *et al*. 2004). A coarse primexine is developed, and no probacula formation is found at the tetrad stage in the *nef1* mutant. Like the *dex1* mutant, the plasma membrane structure in the *nef1* mutant is less invaginated than that in the WT at this stage. The lipid bodies in the mutant microspores are larger than those in the WT. Sporopollenin deposits to the coarse primexine, but never anchors to the primexine and plasma membrane. Instead, a crescent-shaped agglutination of the sporopollenin appears and surrounds the microspores (**Figs. 5A, 5B**). No sign of bacula and tectum formation is found, indicating that the exine development is completely arrested in the *nef1* mutant. Finally, microspores lacking wall formation degrade with considerable fragmentation of the plasma membrane. Also, the amount of the sporopollenin observed on the callose wall, which is putatively derived from tapetum, is significantly reduced compared to the WT at the tetrad stage. Thus it is suggested that the NEF1 functions not only in primexine formation but also in sporopollenin synthesis or deposition. The final size of the plastids of the tapetum is smaller in the *nef1* mutant than in the WT, and lipid accumulation both in plastids and lipid bodies is less observed compared to that in the WT. Further, chloroplast morphology in leaf tissue of the *nef1* mutant has a non-uniform structure compared to that of the WT, and the *nef1* mutant has less granal stacking in the thylakoid membrane. These results indicate that NEF1 is necessary for normal plastid development. The biochemical lipid analysis has shown that the total lipid content examined in the *nef1* mutant is significantly lower than that in the WT. This analysis suggests that normal lipid accumulation or metabolism in the tapetum is indispensable for microspore development including exine pattern formation.

The *NEF1* encodes a protein of 1123 amino acids with very limited sequence similarities to many membrane proteins or transporter-like proteins, and the NEF1 protein is predicted to have a plastid-targeting peptide and 27 transmembrane domains. NEF1 contains prokaryotic membrane lipoprotein lipid attachment sites that are involved in maintaining cell envelope integrity, suggesting that the NEF1 maintains the envelope integrity in the plastids. However, the actual function of the protein, and how the NEF1 is involved in primexine formation have not been elucidated yet. At present, it is suggested that the abnormal lipid accumulation or metabolism in the tapetum results in the production of microspores with excess lipid bodies, which alter the microspore plasma membrane structure and primexine formation. Alternatively, NEF1 might be a plastid envelope protein that functions as a fatty acid transporter. It is suggested that NEF1 imports or exports fatty acid components inside or outside the plastids. The imbalance between the import and export of fatty acids may be caused by NEF1 disruption, which results in the mutant phenotypes (Ariizumi *et al*. 2004).

### *male sterile1* (*ms1*)/*hackly microspore* (*hkm*) mutant

Another example for the primexine mutant is the male sterile *hackly microspore* (*hkm*) of *Arabidopsis* (Ariizumi *et al*. 2005). Normal primexine formation is not observed in the *hkm* mutant at the tetrad stage. Instead, a moderately electron-dense layer is formed around the microspores. Probacula formation is not visible, and the microspore plasma membrane invaginates deeper than that of the WT at this stage. This electron-dense layer is speculated to be the primexine which partially plays a role in sporopollenin deposition onto the microspores, since produced sporopollenin de-

posits to the plasma membrane and the sporopollenin aggregates seem to anchor to the plasma membrane of microspores (**Fig. 5C**; Ariizumi *et al*. 2005). Like the *ms2* mutant, abnormal vacuolation of the tapetum is evident after the tetrad stage, and the vacuolated tapetum collapses the developing microspores. Differentiation of plastid and lipid bodies in the tapetum is not observed in the *hkm* mutant, either. The *HKM* gene is identical to the *MALE STERILITY1* (*MS1*) gene (Dawson *et al*. 1993; Wilson *et al*. 2001; Ito *et al*. 2002; Vizcay-Barrene and Wilson 2006). Subsequent ultrastructural work of the *ms1*/*hkm* mutant by Vizcay-Barrene and Wilson (2006) has shown that the *ms1* immature microspores tend to stick together unlike those of the WT. This suggests that the chemical composition of exine (or exine like structure) of the *ms1* mutant might be altered, possibly due to impaired sporopollenin synthesis and/or secretion from tapetum which does not develop plastids and lipid bodies. The authors reported that primexine formation in the *ms1* mutant (*ms1.1* allele) is similar to that of the WT, although the exine formation is seriously affected. This difference from the *hkm* mutant could be due to the two distinct truncated gene products that are generated by different mutation sites: The *hkm* mutation is located at the 819$^{th}$ nucleotide from the ATG codon, which results in creation of a truncated 18 amino acid and a stop codon at the 876$^{th}$ nucleotide, while the *ms1.1* mutation is located at the 598$^{th}$ nucleotide, and it causes a nucleotide transition from G to A.

The *MS1*/*HKM* gene encodes a 672 amino acid protein with a nuclear targeting signal, leucine zipper motif and PHD-finger motif which is involved in chromatin-mediated gene regulation, suggesting that MS1/HKM is a transcriptiton factor (Wilson *et al*. 2001; Ito and Shinozaki 2002). Actually, it has been demonstrated that an MS1-GFP fusion protein is localized in the nucleus when it is transiently introduced into *Arabidopsis* root cells. By *in situ* mRNA hybridization and *MS1* promoter analysis using GUS (β-glucosidase), it has been shown that the *MS1* gene is expressed in the tapetum from the tetrad stage, and potentially in the microspores at the uninucleate microspore stage, but not in the microsporocyte (Wilson *et al*. 2001; Ito and Shinozaki 2002). Therefore, MS1/HKM may act by regulating transcription of tapetal genes associated with tapetal development and exine formation. Alternatively, based on the fact that tapetal degeneration of the *ms1* mutant does not go through the process of programmed cell death (PCD), the MS1/HKM is suggested to regulate tapetal development by directly regulating tapetal PCD (Vizcay-Barrene and Wilson 2006).

These results suggest that the primexine plays a role in guiding the depositing sporopollenin toward the plasma membrane, and in anchoring the sporopollenin to the plasma membrane. Partial ability for guiding sporopollenin, or sticking sporopollenin to the membrane is observed in the *dex1* and *hkm* mutants, respectively. On the other hand, it has been shown that the *nef1* coarse primexine completely loses these abilities, indicating that the *nef1* primexine is the most dysfunctional among them. Further analysis including the elucidating of the actual protein function of DEX1 and NEF1, and identification of genes downstream of the *MS1*/*HKM* gene will help to better understand the whole mechanism in primexine formation.

### *adenine phosphoribosyltransferase1* (*apt1*) mutant

Unlike *nef1* and *ms1*/*hkm* mutants, the male sterile *apt1* mutant of *Arabidopsis* over-accumulates the lipid components both in the plastids and the lipid bodies in the tapetum, and the size of lipid bodies is approximately 2.7 μm in diameter, which is bigger than those in the WT (approximately 1.9 μm in diameter). Zhang *et al*. (2002) reported that the primexine formation is reduced, and that probacula formation does not occur properly. Microspore development is arrested and finally degraded in the locule. It is suggested that the failure in pollen development is attributed to a lack of the nutrient supply from the tapetum to the developing microspores because of the abnormal lipid metabolism in the tapetum. The *APT1* gene encodes an adenine phosphoribosyltransferase1 which produces AMP from adenine, and is suggested to play a role in maintaining metabolism in the tapetum.

## CALLOSE WALL IS IMPORTANT FOR EXINE PATTERNING

### *callose synthase5* (*cals5*) *mutant/less adherent pollen1* (*lap1*) mutant

The callose wall formation from microsporocytes in the *cals5* mutants (*cals5-1* and *cals5-2*) is almost completely undetectable (Dong *et al*. 2005). Until the microsporocytes go through meiosis, there is no difference in microsporocytic structure between *cals5* and WT. After the *cals5* microsporocytes go through meiosis, they produce tetrads without distinct borders that separate the microspores due to the loss of the callose wall. At the tetrad stage, the plasma membrane structure of the microspores in the *cals5* mutant is wavy and irregular compared to that in the WT. Microspores are released from tetrads, but they are shrunken, broken and eventually degenerate. These released microspores are apparently lacking in sculpturing exine patterning, and exhibit no sign of bacula and tectum formation. Instead, electron-dense globular structures or aggregates, putatively sporopollenin aggregation, deposit on the surface of microspores (Nishikawa *et al*. 2005). The *CSLS5* gene encodes a 1923 amino acid protein with 16 transmembrane domains, GTP-binding motif and an ABC transporter motif. Based on these results, it is predicted that the CALS5 acts as a callose synthase, and may interact with other components of the callose synthase complex (Dong *et al*. 2005).

Several *Arabidopsis* mutants whose pollen grains are defective in adherent ability to stigma have been isolated by employing simple binding assays (Zinkl and Preuss 2000; Nishikawa *et al*. 2005). One of these mutants, the *less adherent pollen1* (*lap1*) mutant, has been shown to be identical to the *cals5* mutant (Nishikawa *et al*. 2005). Nishikawa *et al*. (2005) have also identified three other mutant alleles of *cals5*/*lap1* (*cals5-3*, *cals5-4* and *cals5-5*), and demonstrated that weaker alleles of *cals5-4* and *cals5-5* are lacking in peripheral callose wall unlike stronger alleles of *cals5-3*. However, these two weak alleles still have a significant amount of callose wall between microspores. The stronger allele (*cals5-3*) produces non-viable pollen with complete disruptiton of exine pattern formation, while two weak alleles (*cals5-4* and *cals5-5*) produce viable pollen with an aberrantly patterned exine in which sporopollenin aggregates deposit onto the plasma membrane. This result indicates that a normal amount of callose wall is necessary to produce organized exine pattern formation. The loss of the callose wall may cause a loss of primexine formation, since disorganized aggregates of sporopollenin randomly deposit on *cals5* microspores like *dex1* and *nef1* mutants. The callose wall might be important for trapping primexine subunits around microspores, increasing their local concentration and preventing them from diffusing into the anther locule. Alternatively, it is also possible that the callose wall provides a physical support of primexine assembly (Nishikawa *et al*. 2005). Cytological analysis of the primexine formation of the *cals5* mutants would be intriguing in order to elucidate these possibilities.

### *kompeito* (*kom*) mutant

Another *Arabidopsis* mutant defective in callose wall formation includes a *kompeito* (*kom*) mutant which also shows male sterility (Kanaoka *et al*. 2003). In the *kom* mutant, the callose wall formation is not visible during the meiosis stage. The tetrads are formed, but they apparently have a lower amount of callose wall in comparison to that in the WT. Similar to the *cals5*/*lap1* mutant, sporopollenin aggregation

randomly deposits on the microspores. It is reported that the *KOM* encodes a membrane protein which localizes in the Golgi apparatus and is expressed in the microsporocytes (Kanaoka *et al*. 2003).

### Artificial dissolution of callose wall

It is known that some cytoplasmic male sterile petunia lines show early premature degeneration of the callose wall (Izhar and Frankel 1971). Worrall *et al*. (1992) mimicked this aspect of these petunia lines in transgenic tobacco by introducing a modified pathogenesis-related (PR) β-1,3-glucanase under tapetum-specific *A3* and *A9* promoters. These promoters are shown to activate gene expression from early stages of meiosis before the appearance of endogenous β-1,3-glucanase activity in the locule (reviewed by Scott *et al*. 1991; Paul *et al*. 1992). Although these transgenic plants exhibit early degeneration of the callose wall during meiosis, microspores remain held together as tetrads in spite of the absence of a callose wall. This indicates that the callose wall is not necessary for meiosis. However, produced microspores in tetrads are apparently deformed with a non-compressed multilaminate structure around the microspore surface. The sporopollenin aggregates randomly deposit on the aberrant membrane surface, and the formation of a probacula and tectum is never observed. Finally, the globular sporopollenin aggregates cover the microspores, resulting in a complete lose of exine formation. Subsequent work by Tsuchiya *et al*. (1995) also demonstrated that exine formation is disturbed by early dissolution of the callose wall in transgenic tobacco with PR-type β-1,3-glucanase isolated from soybean under tapetum-specific promoter *Osg6B* which activates gene expression from the tetrad stage (Tsuchiya *et al*. 1994).

## OTHER ARABIDOPSIS MUTANTS DEFECTIVE IN EXINE PATTERN FORMATION

### *male-sterile9* (*ms9*) and *male-sterile12* (*ms12*)

Two exine-defective mutants, *male-sterile9* (*ms9*) and *male-sterile12* (*ms12*), have been isolated by Taylor *et al*. (1998). Normal microspore development is not observed after microspore release from the tetrad in the *ms9* mutant. The produced microspores show a highly vacuolated structure with degenerating cytoplasm and are surrounded by an electron-opaque wall consisting of surface-adhering globular materials. This wall lacks a sculpture structure, and the abnormal microspores finally arrest development. The *ms12* mutant produces microspores with a partially-organized exine. The exine structure of the microspore shows incomplete development of the tectum and bacula. Wall-less microspores are occasionally produced, whereas cytoplasmic organelles and intine formation can be observed. The diameter of these defective microspores is approximately 70 μm, which is 3-4 times larger in size than those of the WT microspores (approximately 20 μm) at the bicellular pollen stage. However, these giant microspores nevertheless undergo developmental arrest at later stages after all. Although these genes have not been identified yet, it is expected that their identification would provide further knowledge of exine pattern formation.

### Downregulation of *Ctr-related copper transporter* (*COPT1*)

Sancenon *et al*. (2004) reported that pollen grains showing a defect in reticulate pattering of surface morphology were obtained in transgenic *Arabidopsis* plants with an antisense construct of the *Ctr-related copper transporter* (*COPT1*) gene fused to the *CaMV35S* promoter. A supplement of an appropriate amount of copper to the transgenic plants would resolve this defect. It has also been shown from *COPT1* promoter-GUS transgenic plants that the *COPT1* gene is expressed in anther tissue and pollen grains, suggesting that copper might be important for pollen wall formation.

## EXINE FORMATION IS A SPOROPHYTICALLY CONTROLLED PROCESS

Genetic studies using exine-defective mutants also provide important evidence that exine formation is basically a sporophytically controlled process, because a segregation ratio of progeny from plants heterozygous for T-DNA insertions (or heterozygous for the recessive mutation) in the *ms2*, *flp1*, *dex1*, *nef1*, *ms1*/*hkm* and *cals5*/*lap1* mutants fits a theoretical ratio of 3:1 (Normal:Defective in phenotype). This indicates that plants heterozygous for the T-DNA insertion (or heterozygous for the recessive mutation) all have normal microspores. This also indicates that these processes in exine pattern formation are sporophytically controlled, and that some sporophytic tissues are absolutely involved in exine formation.

As for sporopollenin synthesis, there is little doubt about the contribution of one of the sporophytic tissues, the tapetum, for the majority of sporopollenin synthesis. As a supporting fact, it has been shown that a number of genes, including the *MS2* gene, associated with lipid biosynthesis and metabolism are expressed in the tapetum regardless of the plant species during exine formation (Aarts *et al*. 1997; Amagai *et al*. 2001; Endo *et al*. 2002; Ito and Shinozaki 2002; Endo *et al*. 2004). The microspore is also a possible supplier of sporopollenin. However, it has been shown that lipid biosynthesis takes place in significant amounts in the pollen grains after the first mitosis, whereas very little takes place at the early stages of microspore development (Evans *et al*. 1992). Therefore it is considered that the majority of sporopollenin is probably derived from the tapetum. The callose wall is secreted from microsporocytes during meiosis, and there is no contribution from individual microspores considering the fact that plants heterozygous for T-DNA produce a normal amount of callose wall. Regarding primexine formation, Fitzgerald and Knox (1995) suggested that the tapetum is not directly involved in any process of primexine formation because of the presence of the callose wall which is suggested to limit permeability to transport between the microspores and the tapetum (Knox and Heslop-Harrison 1970). Therefore, one explanation is that transcripts from one of the sporophytic tissues, microsporocytes, are inherited by every separated microspore of the tetrads, which translates the transcripts into proteins associated with primexine formation at the tetrad stage (reviewed by Scott 1994). However, there is no direct evidence that the tapetum is not involved in the process. Thus the possibility that the tapetum also releases the primexine precursor at the tetrad stage should not be excluded. In this case, such a precursor must penetrate the callose wall and be delivered to the tetrad microspores. Interestingly, it is likely that tapetally derived lipidic bodies and molecules can diffuse freely through the callose wall (reviewed by Piffanelli *et al*. 1998), although it has not been shown whether these primexine constituents actually contain lipidic bodies or molecules. Further analysis is still needed to clarify these possibilities. It is well known that several mutants defective in fatty acid associated genes show reduced pollen viability (Millar *et al*. 1998; Mou *et al*. 2000; Klaus *et al*. 2002; Yu and Benning 2003). It might be interesting to determine whether these mutants produce a normal primexine or not.

Probacula formation occurring in the primexine is also a sporophytically-controlled process. This raises another question as to whether the tapetum or microspore provides sporopollenin as a supplier of probacula formation. Further analysis is still needed to clarify whether both or one of these tissues are responsible for the formation of probacula.

## AN APPROACH TOWARD UNDERSTANDING THE POSSIBLE ROLE OF THE TAPETUM IN EXINE PATTERN FORMATION

Disturbing only tapetum tissue using tapetum-specific promoters would provide further insight into the involvement in exine formation by the tapetum. It has been suggested that the tapetum undergoes PCD when it degenerates after microspore release (reviewed by Wu and Cheng 2000). Recent studies employing transgenic *Arabidopsis* plants with disturbed tapetum development and PCD have been conducted using PCD-associated genes, *Bax* and *AtBI-1*, which are controlled by two different tapetum-specific promoters which activate gene expression at different developmental stages (Kawanabe *et al*. 2006). The *Bax* gene encodes a mammalian proapoptotic protein whose overexpression causes cell lethality in the budding yeast *Saccharomyces cerevisiae* and *Arabidopsis*, while the *AtBI-1* gene encodes Bax inhibitor protein whose overexpression suppresses Bax-induced PCD in *Arabidopsis* (Kawai-Yamada *et al*. 2002). Kawanabe *et al*. (2006) indicated that when *AtBI-1* is overexpressed under the *Osg6B* promoter (*Osg6B::AtBI-1*), which activates gene expression from the tetrad stage to the bicellular pollen stage, the transgenic plants produce microspores defective in exine structure with a shorter bacula and no tectum, whereas when the *AtBI-1* is overexpressed under the *LTP12* promoter (*LTP12::AtBI-1*) which activates gene expression from the unicellular microspore stage to the bicellular pollen stage (Ariizumi *et al*. 2002), the transgenic plants produce fertile pollen grains with normal sculpturing exine. Similar to this, shorter bacula and fewer tectum phenotypes are observed in the transgenic plants in which the *Bax* gene is overexpressed under the *Osg6B* promoter (*Osg6B::Bax*), whereas transgenic plants under the *LTP12* promoter (*LTP12::Bax*) do form a visually normal basal exine structure with bacula and tectum, although this exine structure may have minor defects in function. It appears that tapetum development in transgenic plants with *Osg6B::Bax*, *Osg6B::AtBI-1* and *LTP12::Bax* is severely impaired followed by abnormal vacuolation. These results indicate that less sporopollenin polymerization of exine takes places in the transgenic plants with *Osg6B::Bax* or *Osg6B::AtBI-1* chimeric constructs than in the WT. This could be due to the arrest of sporopollenin supply from the tapetum whose PCD program is disturbed by overexpression of *Bax* and *AtBI-1*. These results also indicate that the arrest of sporopollenin supply derived from the tapetum from the unicellular microspore stage does not severely impair basal structure of the exine. It would be interesting to confirm whether probacula and primexine formation takes place at the tetrad stage in these transgenic plants, which may provide additional evidence of the tapetum contribution to exine pattern formation.

Consistently, defects both in pollen wall formation and tapetum development are synchronously observed in exine mutants and in transgenic tobacco plants in which exine formation is disturbed as described above. Abnormal tapetum vacuolation occurs in the *ms2* and *ms1*/*hkm* mutants during microsporogenesis, and the plastids and lipid bodies in the tapetum are poorly formed in the *nef1* and in the transgenic tobacco plants. The tapetum starts to degenerate in earlier stages in the *ms9* and *ms12* mutants than in the WT. In the *apt1-3* mutant, the lipid accumulation of the plastids and lipid bodies in the tapetum occurs earlier than it does in the WT. These results suggest that normal tapetum development is important for normal exine pattern formation.

## PLASMA MEMBRANE STRUCTURE IS RELATED TO PROPER EXINE PATTERNING

The factor that determines pollen wall patterning might be included in the plasma membrane. Sheldon and Dickson (1983) employed a centrifugation experiment that enables displacement of cytoplasmic components of meiocytes by the centrifugation of developing anthers. This centrifugation experiment has demonstrated that reticulate exine patterning is apt to be impaired when meiocytes at an early meiotic prophase are used, while the exine patterning is not apt to be disturbed after the centrifugation experiment when meiocytes at the later stages are used. A family of coated vesicles (CVs), which are present throughout the meiocyte cytoplasm, are likely to be associated with large assemblages of smooth endoplasmic reticulum and with the plasma membrane, and it seems that the CVs are responsible for reticulate patterning. It is suggested that the CVs appear at the beginning of meiosis, when they are still sensitive to the centrifugation experiment, and once CVs are progressively inserted into the plasma membrane during meiosis, they become resistant to centrifugation.

It appears that the microspore plasma membrane structure is disturbed throughout the exine-defective mutants as described above. For example, the *dex1* and *nef1* mutants have a shallower membrane structure than that of the WT (Paxson-Sowders *et al*. 2001; Ariizumi *et al*. 2004), whereas the *hkm* membrane is deeper than that of the WT (Ariizumi *et al*. 2005). The membrane of *cals5* is irregularly waved compared to that in WT (Dong *et al*. 2005). It would be interesting to determine whether these CVs are intact in these mutants or not.

## FURTHER ELUCIDATION OF EXINE DEVELOPMENTAL PROCESSES

Genetic research employing *Arabidopsis* mutants has demonstrated that exine pattern formation is dependent on three developmental processes, sporopollenin synthesis, primexine formation and callose wall formation, and that genes associated with these processes have been identified (**Fig. 6**). A lack of any one of these factors results in the pro-

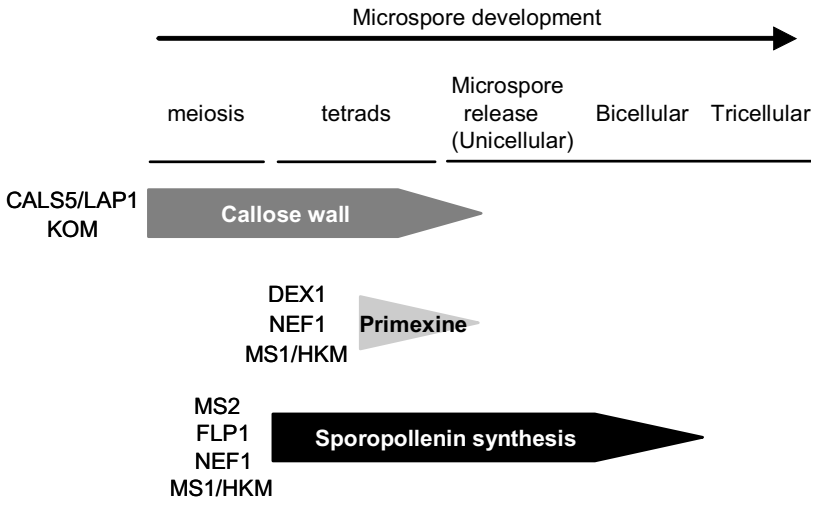


**Fig. 6 Three major developmental processes and related proteins during microspore development.** Callose wall is evident in locule from meiosis. Callose wall dissolution by callase activity begins at the end of tetrad stage. CALS5/LAP1 and KOM are involved in this process. Primexine formation starts at the late tetrad stage. Primexine disappears almost at the same time as callose dissolution. DEX1, NEF1 and MS1/HKM are involved in this process. Sporopollenin synthesis probably starts from early tetrad stage. Sporopollenin synthesis and deposition halts as a result of tapetum degradation. MS2, MS1/HKM and NEF1 are involved in this process at an earlier stage, while FLP1 may be involved in a later polymerization stage.

duction of microspores with an aberrant exine structure even though the other two factors are intact. However, much remains to be clarified by molecular and biochemical approaches, for instances, the functions of the DEX1 and NEF1 proteins, and their subcellular localization in the tapetum or microsporocyte.

Biochemical analysis has demonstrated the constituents of sporopollenin, although it has not been clearly shown what the constituent of primexine is. Neither has it been shown how sporopollenin precursors are carried from the tapetum. Several monocot plants such as rice and wheat have Ubisch bodies (orbicules) that are osmiophilic entities. They are thought to transport tapetal metabolites to the developing exine (reviewed by Piffanelli *et al*. 1998). RAFTIN proteins that are present in Ubisch bodies are important for transferring tapetum metabolites in these monocot plants (Wang *et al*. 2003). Lipid transfer proteins (LTPs) are suggested to play a role in the transfer of tapetal metabolites in *Arabidopsis* (reviewed by Pineffelli *et al*. 1998). At least fifteen lipid transfer proteins exist in the *Arabidopsis* genome (Arondel *et al*. 2000). It would be intriguing to identify a knock-out mutant in which some of the LTP proteins expressing in the tapetum are disrupted.

Exine pattern formation is highly species-specific, and it is a highly conserved developmental process in the plant kingdom, suggesting that highly sophisticated genetic regulation is conserved. Identification of *Arabidopsis* homologous genes from different species may be available because of recent advancements in the genomic database for several plant species. Further genetic approaches by gathering additional exine-defective mutants will provide us not only new insight into the detailed mechanism of exine pattern formation, but also a mechanism of exine patterning in other plant species.

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