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Forging the pollen fortress: Cell biological mechanisms of exine formation

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During its development, pollen becomes surrounded by a complex cell wall known as the exine. Exine is preceded by the primexine–a thin, transient extracellular structure essential for the formation of a well-developed exine but challenging to visualize and study. Exine formation requires a partnership between the developing pollen and the inner sporophytic anther layer, the tapetum. The tapetum produces enzymes and materials necessary for exine development, which are delivered to the surface of developing pollen and become assembled into the distinct layers and patterns of exine. However, how exine materials are transported, and how the events occurring in the tapetum and in developing pollen are coordinated, remains poorly understood. This review highlights recent advances in understanding primexine structure and composition, the trafficking of exine materials toward the pollen surface, and the recently discovered communication mechanism involving the tapetum, developing pollen, and the middle layer of the anther.

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Introduction

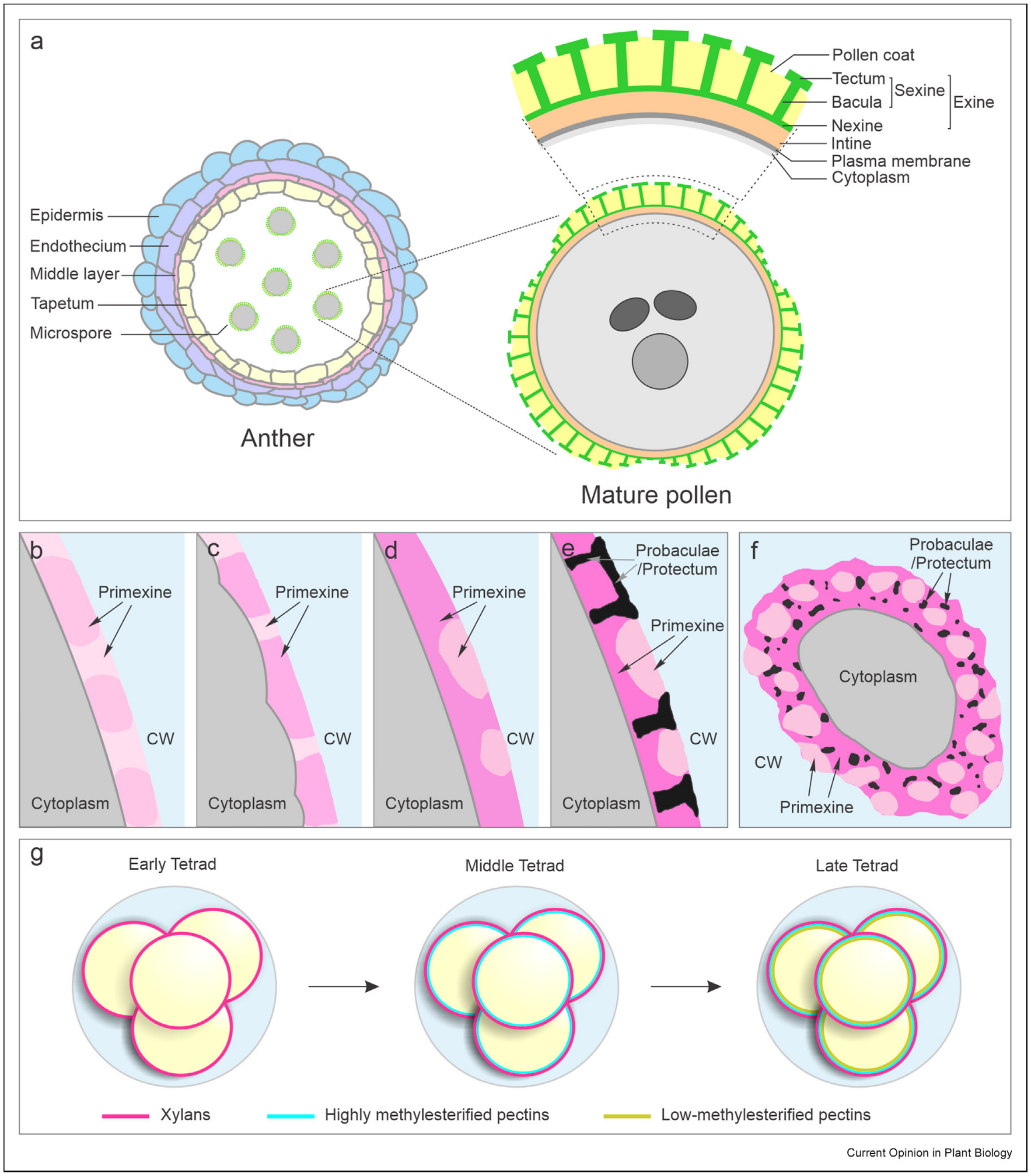
Pollen grains, used by seed plants to deliver sperm cells to female structures, are surrounded by a specialized cell wall, the exine. Exine protects pollen from external and internal stresses and mediates interactions with pollinators and female stigmas. Defects in exine formation are often linked to defects in male fertility and, in many cases, lead to pollen lethality.

As a plant cell wall, exine is highly unusual. Its main component is sporopollenin, a complex biopolymer often described as the toughest material in the plant world [1]. Genetic, chemical, and biochemical evidence suggests that sporopollenin is composed of aliphatic polyketide and phenolic derivatives [1–4]. Advanced analytical techniques have recently provided deeper insights into its composition and structure. In pine, sporopollenin has been proposed to consist of a polyketide-derived polyvinyl core, crosslinked through an acetal moiety with 7-O-p-coumaroylated C16 aliphatic units [2]. In *Brassica*, it has been suggested to contain a core of polymeric phenylpropanoids crosslinked by hydroxylated fatty acids, with additional attachment of other fatty acids and hydroxyl-containing compounds such as naringenin and β-sitosterol [4].

Exine formation involves several essential steps occurring at the surface of developing pollen: callose wall synthesis, plasma membrane undulation, primexine formation, callose wall degradation, and sporopollenin deposition. These steps are tightly coordinated and regulated through a unique partnership between developing pollen and the surrounding tapetal layer. The tapetum produces most of the materials necessary for exine development, which then must be transported to the surface of developing pollen grains [5]. Exine typically exhibits a stratified structure consisting of three distinct layers: the tectum, baculae, and nexine (Figure 1a). The roof-like tectum and the pillar-like baculae contribute to the formation of species-specific patterns on the pollen surface, which, in some species, can be very elaborate [6].

In the last fifteen years, there has been a tremendous progress in our understanding of genetic and molecular mechanisms of exine formation, largely from studies in Arabidopsis and rice, with studies in maize quickly catching up [7–12]. Most significantly, the studies revealed a conserved network of tapetal transcription factors that regulate the expression of a conserved set of enzymes involved in sporopollenin biosynthesis. Detailed descriptions of these mechanisms are available [5,13–16]. In this review, we highlight recent advances in unraveling cell biological mechanisms contributing to exine formation, particularly the roles

Figure 1



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(a) Diagrams of the anther chamber, within which pollen grains develop, and of the pollen wall. The cell layers of the anther chamber and the components of the pollen wall in the mature pollen grain are indicated. **(b–f)** The primexine, developing between the plasma membrane and the callose wall (CW) in late tetrad-stage microspores, is depicted in shades of pink. The probaculae and protectum, the prototypical elements of the future exine, are depicted in black. In Arabidopsis, the primexine forms distinct darker and lighter structures that gradually assemble into the reticulate pattern characteristic of exine in this species. The exine elements initially form at the peaks of the darker material. **(g)** The order of accumulation of different carbohydrate materials within the primexine during the tetrad stage of development is illustrated. The callose wall is shown in blue.

of callose wall and primexine, mechanisms of trafficking within and outside of the tapetum, and communication between the tapetum and other cells in the anther.

Callose wall and primexine: creating the proper environment for exine deposition

The first signs of exine formation become apparent at the tetrad stage of pollen development. In each tetrad, four sister microspores, which will develop into four pollen grains, are enclosed within a shared callose wall. Each microspore in a tetrad will then form a thin extracellular structure, the primexine, between its plasma membrane and the callose wall [17,18].

Mutants in Arabidopsis that are defective in callose wall formation produce exine lacking the characteristic reticulate pattern, instead showing globular sporopollenin deposits [19,20]. This suggests that the callose wall acts as a mold, guiding the proper development of exine. The timing of callose degradation is also important: both premature and delayed degradation disrupt exine patterning [21,22]. The regulation of this timing has recently been linked in Arabidopsis to the transcription factors MYB80 and transposable element silencing via AT-hook (TEK), which control the expression of A6 family callases (β-1,3-glucanases) in the tapetum [22].

Compared to the callose wall, which is thick, longer lasting. and readily observable with either transmission electron microscopy (TEM) or with light microscopy, primexine is much more enigmatic: it is very thin (in Arabidopsis reaching only ~0.1 μm when fully developed), has low electron density, and exists only briefly. Chemically fixed TEM specimens often develop artifacts at the position of primexine, complicating its analysis. However, it has been suggested previously that primexine contains carbohydrate-based molecules [17,23,24].

Despite its slim and transient nature, this layer plays a key role in exine formation, providing the environment for probaculae and protectum, the prototypical elements of future exine, to develop [17,25]. After the callose wall is dissolved and the microspores are released, these elements expand significantly due to the massive deposition of sporopollenin. In both Arabidopsis and rice, mutants with defective primexine do not produce normal exine, and their pollen often degrades soon after the microspores are released from the tetrads [26–30]. These mutants also often accumulate large inclusions of sporopollenin near the surface of microspores or within the anther locule, suggesting that, without the normal primexine, exine materials cannot anchor properly to the pollen surface [26–30].

Recently, a detailed TEM analysis of primexine development in Arabidopsis provided strong evidence that primexine acts as a scaffold for exine formation [31]. The study was performed on samples prepared with high-pressure freezing/freeze substitution, which better preserves the ultrastructure of primexine compared to chemical fixation. At early stages of development, the primexine already displayed two qualitatively distinct compartments, with, respectively, darker and lighter structures that eventually assembled into a reticulate pattern matching the pattern of exine in that species (Figure 1b–f). At the end of the tetrad stage, the probaculae and protectum developed at the peaks formed by the darker material of primexine (Figure 1e–f). The mechanism underlying the regular segregation of darker and lighter structures within primexine remains unclear. However, self-organization based on chemical properties of components—via phase separation or micelle formation—has been proposed as a possible explanation for primexine and exine formation [32,33].

Primexine composition has also been investigated with antibodies recognizing several carbohydrate moieties [31]. Primexine was found to undergo dynamic changes throughout the tetrad stage, accumulating different carbohydrates at specific time points. Xylans appear first in the early tetrads, followed by highly methylesterified pectins in the mid-stage tetrads, and by pectins with low methylesterification appearing in late tetrads (Figure 1g). The presence of punctate signals for these carbohydrates inside the tetrad-stage microspores before they appear in the primexine suggests that these primexine materials are synthesized by the microspores themselves, and not by the tapetum, which synthesizes and exports many of the factors needed to produce functional exine.

Tapetum: the trafficking pathways for sporopollenin precursors

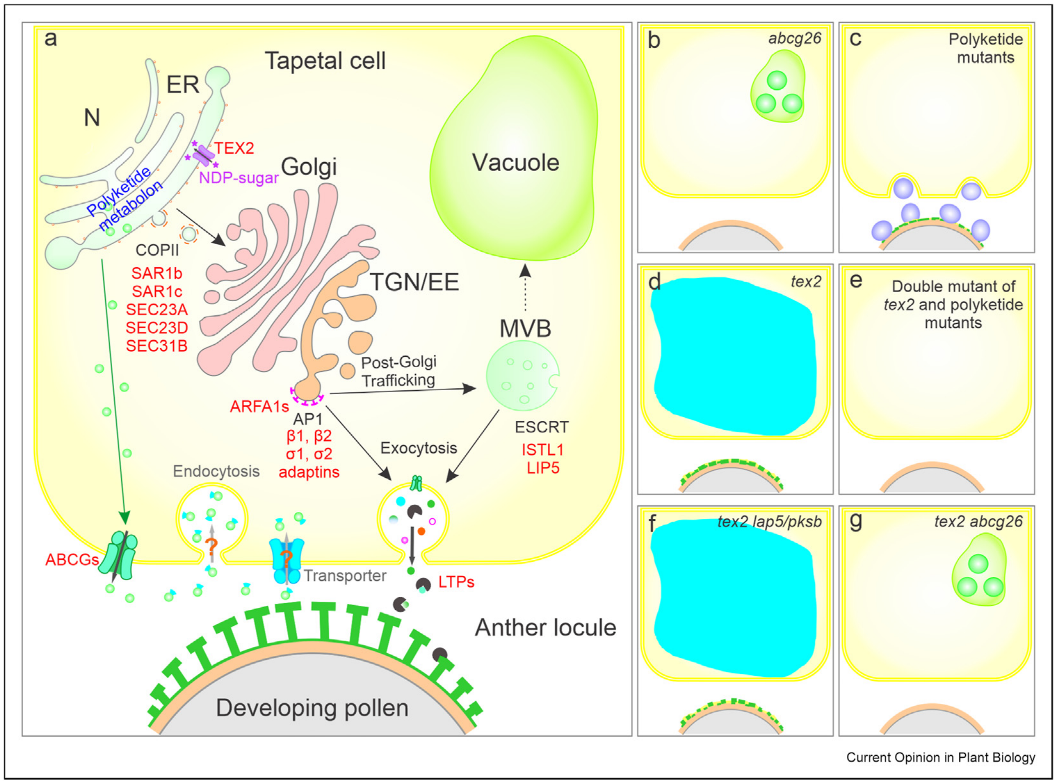
Tapetum, the innermost sporophytic layer of anthers, lines the anther locules where pollen grains develop. It plays a critical role in exine development, synthesizing sporopollenin precursors and transporting them to the surface of developing pollen. The tapetum also transports nutrients, minerals, enzymes, and pollen coat materials to developing pollen [5]. Consistent with its role in producing and transporting large amounts of materials toward the developing pollen, tapetal cells are strongly polarized.

Signs of tapetal polarity include the degradation of the cell wall, which begins at the locular side of the tapetum [18], the formation of plasma membrane evaginations on this side [18,34], and the preferential accumulation of the multivesicular bodies (MVBs) near the plasma membrane facing the locule [35]. Certain transporters, like OsABCG15, and transported materials, like xylan, also localize preferentially to this side [31,36]. In species that produce orbicules/Ubisch bodies (small circular structures believed to carry sporopollenin or its precursors in multiple plant families including the Poaceae, but not in the Brassicaceae), these structures also form on the locular side. In contrast, a phenolics-based apoplastic barrier, the peritapetal strip, forms between the tapetum and the overlying middle layer, ensuring that most transport from the tapetum is directed toward the developing microspores [37].

How the transport of various compounds is organized both within the tapetum and outside of it in the anther locule is still poorly understood. Fatty acid-derived polyketides, key sporopollenin precursors, are produced by a group of enzymes, several of which function together as a metabolon in the endoplasmic reticulum (ER) [38–40] (Figure 2a). Polyketides, or their derivatives, are likely transported to the plasma membrane and then secreted via the plasma membrane-localized ATP-binding cassette transporter ABCG26 in Arabidopsis [41–43] (Figure 2a) and its homologs in rice [44–46] and maize [47]. In support of this model, loss-of-function *abcg26* mutants accumulate abnormal autofluorescent inclusions in tapetal vacuoles and fail to develop exine [43] (Figure 2b). Formation of these inclusions depends on the presence of the functional polyketide metabolon, consistent with ABCG26 exporting polyketide-derived compounds, although its exact substrates remain unknown.

Recent studies have provided compelling evidence that phenolic building blocks produced by tapetal enzymes are also incorporated into sporopollenin [2–4,48], raising questions about how these compounds are

Figure 2



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A model of the secretory pathways in the tapetum that transport proteins and materials for exine formation. **(a)** Proteins whose roles in tapetal trafficking have been confirmed by the mutant analyses in Arabidopsis are highlighted in red. Membrane proteins such as ABCG26 and secreted proteins such as lipid transfer proteins (LTPs) are likely trafficked via the anterograde trafficking pathway, which in the tapetum also involves the endosomal sorting complex required for transport (ESCRT) proteins ISTL1 and LIP5. Polyketide derivatives produced in the endoplasmic reticulum (ER) (small green circles) may be released into the cytoplasm and subsequently exported to the anther locule via the ABCG26 transporter, or they may undergo further modifications within the endomembrane system before being export. Based on the analysis of the *tex2 abcg26* double mutant, the materials exported by ABCG26 may return to the tapetum–possibly after undergoing modification in the locule–to form more mature sporopollenin precursors. This process is depicted by combined green circles and blue triangles, which are returned to the tapetum either via an unknown transporter or through endocytosis. Subsequent modification or later export of these precursors involves a TEX2-dependent step. ER, endoplasmic reticulum; MVB, multivesicular body; N, nucleus; TGN/EE, trans-Golgi network/early endosome.**(b–g)** Inclusions accumulating in or near the tapetum in several Arabidopsis mutants may help elucidate how the tapetal transport of sporopollenin precursors is organized. **(b)** In the *abcg26* mutant, fluorescent inclusions dependent on the polyketide biosynthesis accumulate in tapetal vacuoles, while pollen fails to develop exine and degenerates. **(c)** In the polyketide biosynthesis mutants, a different type of inclusions—proposed to contain phenolic compounds—accumulates just outside the tapetal plasma membrane and around the surface of the microspores. Exine formation largely fails in these mutants, although some mutants develop a thin exine. **(d)** In *tex2* mutants, very large inclusions containing sporopollenin-like materials accumulate in the tapetal cells, and pollen develops a very thin exine. **(e)** Accumulation of tapetal inclusions in *tex2* is suppressed by mutations in most polyketide biosynthesis genes. Pollen degenerates in most of these double mutants, even though it survives in the single mutants. This suggests that the double mutations block more than one biosynthetic or trafficking pathway for exine formation. **(f)** The *lap5/pksb* mutations fail to suppress the accumulation of the *tex2* inclusions. Single mutants of *LAP5/PKSB* also accumulate similar inclusions. **(g)** In the *tex2 abcg26* double mutant, the accumulation of the *tex2* inclusions is suppressed, but the characteristic *abcg26* vacuolar inclusions still develop.

trafficked out of the tapetum and at what point they become combined with polyketide derivatives. Interestingly, autofluorescent inclusions proposed to contain phenolics, such as hydroxycinnamoyl spermidine, were found to accumulate in Arabidopsis mutants defective in polyketide biosynthesis [43] (Figure 2c). Electron microscopy analyses showed that these inclusions were fairly small (~0.5 μm in diameter), possibly membraneless, mostly electron-lucent with some electron-dense regions, and located outside of tapetal cells—often in close proximity to all sides of the tapetal plasma membrane or near the surface of microspores [43]. These findings suggest that phenolics might be trafficked out of tapetum through a pathway different from polyketides, although they depend on the presence of polyketides for incorporation into sporopollenin, which might happen either on the microspore surface or earlier. VAMP726, a member of the family of vesicle-associated membrane proteins (also known as R-type SNAREs) that typically mediate fusion of vesicles carrying proteins or other compounds with other membranes [49], has been recently shown to influence the phenolic content of sporopollenin in Arabidopsis and maize [50], although it is unclear whether it does this by facilitating trafficking of phenolics themselves, of proteins involved in incorporation of phenolics into sporopollenin, or of something else.

THIN EXINE2 (TEX2) is another Arabidopsis protein implicated in trafficking sporopollenin precursors out of tapetum [51]. TEX2 is an ER-localized protein belonging to the family of nucleotide sugar transporters, which usually import monosaccharides into the ER and Golgi lumens, where they glycosylate proteins and lipids or form polymeric carbohydrates. However, the substrate of TEX2 is still unknown.

tex2 mutants also accumulate tapetal inclusions [51]. These inclusions, however, differ greatly from those found in either *abcg26* mutants or in polyketide biosynthesis mutants. They are much larger, often occupying entire cells, have an amorphous structure, and share significant similarities with the sporopollenin of mature exine (Figure 2d). Accumulation of these inclusions in *tex2* depends on the activity of most polyketide biosynthesis enzymes (Figure 2e) — with the curious exception of LAP5/PKSB (Figure 2f), whose single mutant (but not that of its paralog LAP6/PKSA) was also found to accumulate similar inclusions [51]. The *tex2* inclusions also require functional ABCG26. While the loss of ABCG26 prevents the accumulation of *tex2* inclusions, the loss of TEX2 does not affect the accumulation of the *abcg26* inclusions (Figure 2g), suggesting that the process mediated by TEX2 acts downstream of the ABCG26 transport.

The fact that accumulation of *tex2* inclusions requires functional ABCG26 is particularly intriguing. This observation might suggest that the compounds accumulating in *tex2* represent more mature precursors of sporopollenin compared to those accumulating in *abcg26*, and that the compounds exported by ABCG26 undergo modification in the anther locule and then perhaps re-enter tapetal cells to be further modified or exported through a mechanism involving TEX2 activity.

Several tapetal proteins associated with the anterograde trafficking pathway have also been recently shown to be important for exine formation in Arabidopsis (Figure 2a). This includes proteins involved in the formation of the COPII vesicles that mediate transport from ER to Golgi (SAR1b, SAR1c, SEC23A, SEC23D, and SEC31B) [52–54] and proteins involved in Golgi and post-Golgi anterograde trafficking (small GTPases of the ArfA1 class and several subunits (referred to as adaptins) of the heterotetrameric complex ADAPTOR PROTEIN-1 (AP-1), which facilitates the recruitment of cargo and coat proteins during clathrin-coated vesicle formation in the trans-Golgi network/early endosome) [55–57]. In COPII mutants, sporopollenin-like electron-dense materials accumulate inappropriately between the tapetum and the overlying middle layer or in the locule, without attaching to the microspore surface [52–54]. Thus, these mutations, while not blocking the secretion of these materials, likely affect the polarity of tapetal transport and possibly the secretion of factors necessary for the movement of these materials through the locule and their attachment to the microspore surface.

Interestingly, the endosomal sorting complex required for transport (ESCRT) components ISTL1 and LIP5, which in vegetative cells are involved in the endosomal sorting via MVBs and the directing of endocytosed proteins to vacuoles for degradation [58], participate in the exocytosis of plasma membrane and secreted proteins in the tapetum [35] (Figure 2a). This suggests that ESCRT proteins may be recruited to perform specialized and unique functions in the tapetum to support its high secretory activity. The structure of tapetal MVBs is affected in the *ist1 lip5* double mutant: compared to the MVBs in the wild-type tapetum, the mutant ones are smaller, contain fewer intraluminal vesicles, and these vesicles fail to form concatenated structures [35].

Delivery of the ABCG26 transporter to the plasma membrane most likely depends on the anterograde vesicle trafficking, although none of the studies mentioned above have explicitly examined whether ABCG26 localization changes in the corresponding mutants. However, some other members of the ABCG family expressed in the tapetum failed to localize to the plasma membrane in several of the trafficking mutants [35,52,57]. Additionally, secretion from the tapetum of type III lipid transfer proteins (LTPs), which may carry sporopollenin precursors through the anther locule [59], is disrupted in the *ap-1* and *istl1 lip5* mutants [35,56].

Trafficking through the anther locule

How various molecules secreted by the tapetum are transported through the anther locule toward the microspores remains a mystery. In many plants, including Arabidopsis, microspores are often not in direct contact with the tapetum. This implies that secreted materials must traverse the locular space, which can be quite substantial. Microspores are surrounded by the locule fluid, secreted by the tapetum. This fluid appears lattice-like under cryo-scanning electron microscopy (cryo-SEM) in cryo-fractured anthers, suggesting that structure of the locule may facilitate the trafficking of materials to the microspores [34].

Since fatty acid derivatives like polyketides are part of sporopollenin, LTPs are expected to play a role in their journey across the locule. In Arabidopsis, two type III LTPs are expressed by the tapetum during the tetrad stage (stage 7 of anther development) and secreted into the locule during early and middle exine formation stages (stages 8–9) [59] (Figure 2a). These LTPs then accumulate on the microspore surface during exine maturation (stages 9–10). Interestingly, although these LTPs are expressed and secreted normally in mutants defective in polyketide biosynthesis and ABCG26-dependent transport, they fail to accumulate on the microspore surface. This indicates that polyketides are necessary for this step [59]. It remains unclear whether these LTPs bind polyketides directly or carry other precursors that rely on polyketides for anchoring to the developing exine.

Type III LTPs from a grass, triticale, have also been shown to be specifically expressed in the tapetum and exported to the anther locule during exine formation [60]. Additionally, in rice, the tapetal LTP OsC6, which belongs to another class of LTPs, is important for orbicule formation [61]. When the OsC6 expression was downregulated, the number of orbicules was greatly reduced. Recently, another tapetal LTP in rice, the plasma membrane-embedded LTP47, was shown to interact with OsC6, suggesting that these lipid-binding proteins might collaborate in the orbicule formation [62].

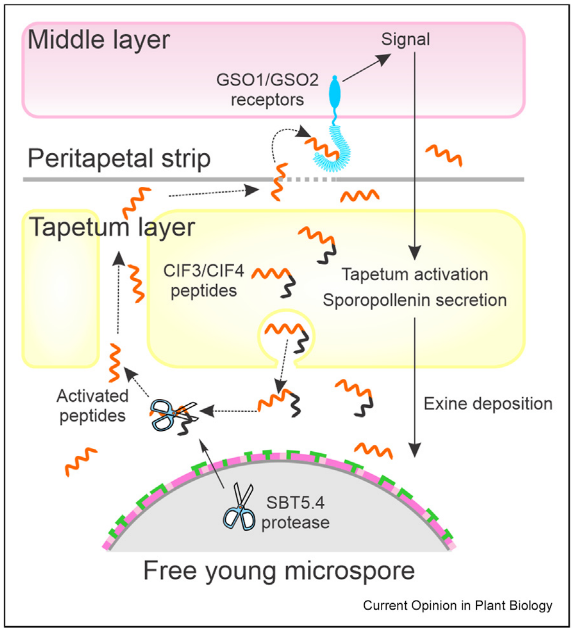
Communication between anther layers coordinates exine formation

Exine formation requires partnership between the tapetum and microspores, necessitating effective interlayer communication to coordinate their development and activity. A recent study, based on the analysis of Arabidopsis mutants and the expression patterns of the corresponding genes, has identified one mechanism for such communication [63]. Interestingly, this mechanism also involves a third cell layer—the middle layer that lies above the tapetum.

According to the proposed model, cells of the middle layer express transmembrane receptor-like kinases GASSHO1 (GSO1) and GSO2, while the tapetum produces precursors of secreted peptides called CASPARIAN STRIP INTEGRITY FACTOR 3 (CIF3) and CIF4, which act as ligands for GSO1/2 receptors. Activation of these peptides requires cleavage by proteases, such as the subtilase SBT5.4, which is expressed in developing microspores. Notably, the onset of protease expression coincides with the beginning of exine deposition.

The model suggests a complex journey for the CIF3/4 peptides (Figure 3). First, as precursors, they travel from the tapetum toward the microspores and are cleaved by proteases. Then, the active peptides must travel back to the middle layer to bind GSO1/2 receptors. The stage-specific production of the peptide-cleaving proteases is thus predicted to act as a trigger for the signaling pathway in the middle layer, which in turn activates secretion of sporopollenin components from the tapetum.

Figure 3



A model, based on Ref. [63], illustrating interlayer communication that coordinates exine formation. The precursors of the CASPARIAN STRIP INTEGRITY FACTOR (CIF) peptides are produced by the tapetum and secreted into the anther locule. These precursors are then cleaved by proteases, such as SBT5.4, which are produced by the microspores near the onset of exine deposition. It remains unclear if the CIF precursors are cleaved in the anther locule or after reaching the microspore surface. The activated CIF peptides subsequently travel to the middle layer and bind to the GSO receptors on its surface. This binding is expected to initiate downstream signaling processes in the middle layer, which in turn trigger the release of sporopollenin materials from the tapetum.

The model raises multiple questions. How is the complicated transport process of the CIF3/4 peptides organized? The peritapetal strip, an apoplastic barrier that forms between the middle layer and the tapetum during the meiotic stage [30] (Figure 3), adds further complexity, as the peptides must cross this barrier. Additionally, what signals are produced downstream of the activated GSO1/2 receptors in the middle layer? How are these signals transported through the peritapetal strip, and what signaling pathway in the tapetum responds to them?

Concluding remarks

Although many exciting details about exine formation have been uncovered in recent years, many questions remain. While primexine clearly provides a critical environment for developing exine elements and serves as a scaffold for exine deposition, its composition is still not fully understood. Moreover, how the different components of primexine align with the lighter and darker structures is unknown. The composition of primexine should be studied across multiple species to determine whether species-specific differences in primexine specify variations in exine patterns. Additionally, although it is likely that multiple components of primexine originate from the microspores, the contribution of the tapetum to its formation cannot be ruled out. If some primexine compounds are indeed derived from the tapetum, it remains unclear how they are transported across the thick callose wall.

In general, the organization of exine material and protein transport remains mysterious. The proposed model of communication among the tapetum, microspores, and the middle layer [63] suggests that there could be interesting and sophisticated trafficking mechanisms yet to be discovered. Both time-lapse live-cell imaging and pharmacological interventions, two essential tools of cell biology, have not yet been employed for visualizing and studying trafficking in the tapetum and the locule. Because the tapetum and the developing microspores are located deep within the anthers, these methods are challenging to apply to these tissues. However, as more cell type-specific promoters are identified through genetic studies of exine-related genes, the application of these tools, particularly live imaging, may become more available for addressing these questions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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* * of outstanding interest

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This study evaluated various methods for sporopollenin dissolution and found that ethanolamine effectively dissolves sporopollenin of seed plants but not that of early-evolving plant lineages. Based on several chemical assays, the authors proposed a structural model for sporopollenin from *Brassica napus*, in which polymeric phenylpropanoid derivatives are crosslinked by hydroxylated fatty acids, form the core structure.

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This study discovered a signaling mechanism involving a three-way communication among the receptors on the surface of cells in the middle layer, the ligands produced by the tapetum, and the proteases produced by microspores, which are required for the timely activation of these ligands. The mechanism is proposed to coordinate tapetum activity with pollen development, ensuring that the formation of pollen wall components in the tapetum occurs precisely when the developing pollen is ready to receive them.