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EXINE AND APERTURE PATTERNS ON THE POLLEN SURFACE: THEIR FORMATION AND ROLES IN PLANT REPRODUCTION

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Abstract: Pollen grains, the male gametophytes of seed plants, surround themselves with a complex pollen wall for protection from various environmental stresses. The deposition and assembly of exine, the outer layer of the pollen wall, lead to the formation of patterns on the pollen surface that are species specific, tremendously diverse, and often very beautiful. These patterns arise due to exine's assembly into various nano- and microstructures, and due to the absence of exine deposition at certain areas of the pollen surface. The areas that have reduced exine deposition, or lack it completely, are known as pollen apertures, and their patterns are also species specific and highly variable. Although the intricate patterns of exine and apertures have been drawing attention for centuries, it is still not clear how exactly they develop, what genes are involved in their formation, and what purpose they serve. Here, we review the current state of knowledge about the exine and aperture patterns, their perceived roles in plant reproduction, and the cellular and molecular mechanisms that guide their formation.

Keywords: pollen, exine, aperture, pollen wall, microsporogenesis, exine pattern, aperture pattern, membrane domain, sporopollenin

1 Patterns on the Pollen Surface

Pollen grains of seed-producing plants act as specialised vehicles, delivering immotile sperm cells to female structures. Pollen, therefore, plays a key role in plant reproduction, – and it performs this role in style. In many species, the pollen surface develops intricate and complex patterns formed by deposition

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of the pollen wall exine (Figure 1) (Kesseler and Harley, 2004; PalDat, 2017). Formation of these pollen patterns has been referred to as 'a *tour de force* of biological morphogenesis' (Heslop-Harrison, 1971).

Starting with Nehemiah Grew in the seventeenth century, believed to be the first to describe pollen (Grew, 1676, 1682; Manten, 1969), beautiful patterns on the pollen surface have long been admired by microscopists, plant physiologists, botanical illustrators, palynologists, evolutionary biologists, and, more recently, molecular geneticists (Purkinje, 1830; Fritzsche, 1837; Wodehouse, 1935; Erdtman, 1943; Edlund et al., 2004; Kesseler and Harley, 2004; Suzuki et al., 2008; Dobritsa et al., 2011). The details and ornamentations that make up these patterns are on the order of several micrometers to less than 100 nm. They are best visualised with the help of high-magnification/high-resolution microscopy (e.g. electron or confocal), but in some cases these can be recognised even with low-magnification dissecting microscopes. The palynological database PalDat (www.paldat.org) at the University of Vienna, Austria, provides free access to thousands of high-quality SEM and TEM pollen images from a large number of plant taxa, serving as an excellent resource for anyone interested in morphology of pollen walls (PalDat, 2017).

Pollen patterns can differ tremendously in their appearance (Figure 1), making the pollen surface one of the most diverse microstructures in nature, on par with other beautiful and diverse microscopic patterns formed by cell walls of diatoms and skeletons of radiolarians (Anderson, 1983; Sumper, 2002; Gordon et al., 2009). Individuals of the same species usually produce pollen with the same pattern, and closely related plant species often produce similarly patterned pollen. Yet, within the same family – or occasionally even within the same genus (Banks and Rudall, 2016) – there could also be differences in patterns, sometimes very significant. Because of the tremendous diversity of its surface patterns, pollen is routinely used in taxonomic classifications (Naik, 1984) and in forensic analysis (Horrocks et al., 1998; Mildenhall, 2004, 2006). To describe the enormous variety of patterns on the pollen surface, palynologists have created highly complex terminology (Punt et al., 2007; Hesse et al., 2009), referring to pollen patterns as microechinate, foveolate, striato-reticulate, and anazonasulculate, among many others. Several illustrated glossaries (Halbritter et al., 2006; Punt et al., 2007; Hesse et al., 2009) represent attempts to make pollen terminology more accessible to beginners and non-specialists.

2 Why Do Pollen Walls Assemble in Complex and Diverse Patterns?

The variety of intricate patterns on the pollen surface led to long-standing questions of why these patterns exist in such a diverse array. Is there a

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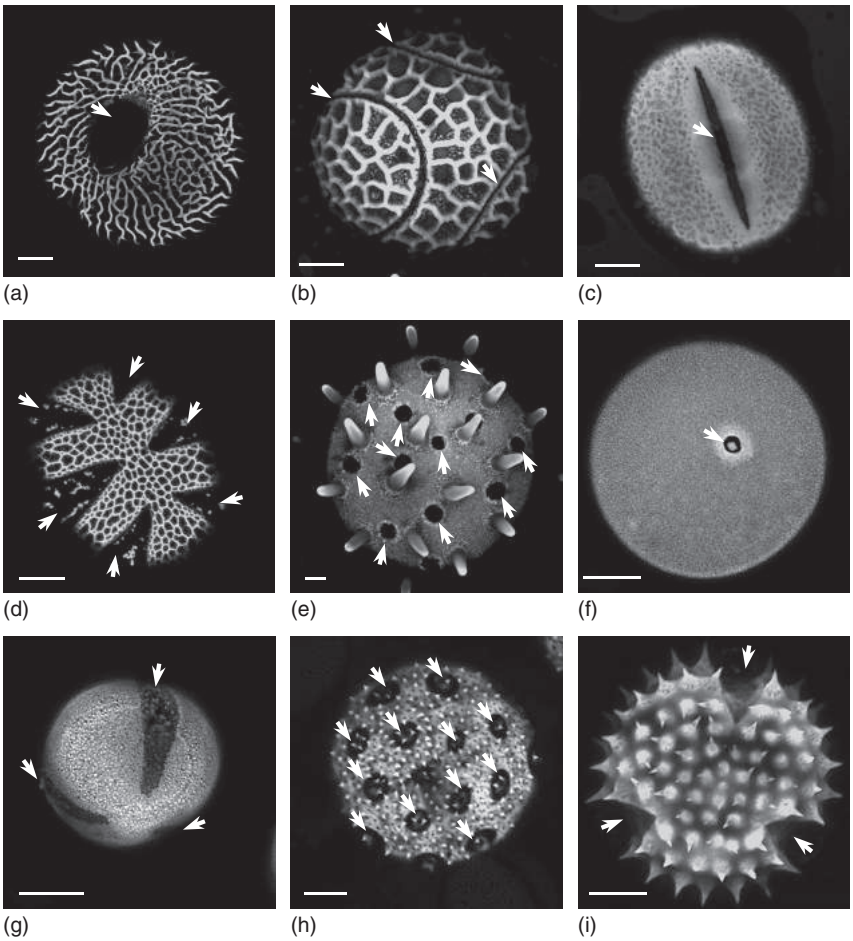

Figure 1 Exines assume beautiful and often dramatically different patterns on pollen surfaces across plant taxa. Confocal images of pollen from species belonging to several plant families. Pollen grains are stained with the fluorescent dye auramine O that binds to exine. Apertures (arrows), the areas where exine is not deposited or is underdeveloped, represent a common patterning element of pollen surfaces and can vary dramatically in different species in number and morphology. (a) *Pelargonium hirtum* (Geraniaceae); (b) *Passiflora* sp. (Passifloraceae); (c) *Pisum sativum* (Fabaceae); (d) *Salvia patens* (Lamiaceae); (e) *Hibiscus schizopetalus* (Malvaceae); (f) *Panicum virgatum* (Poaceae); (g) *Gloxinia sylvatica* (Gesneriaceae); (h) *Silene alba* (Caryophyllaceae); (i) *Tagetes* sp. (Asteraceae). An operculum is visible in the middle of the aperture in (F). Scale bars = 10 μm.

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functional significance behind the myriad ways the pollen walls assemble? Do evolutionary pressures drive pattern diversification? Or is the differential assembly of pollen walls a byproduct of differences in their biochemistry?

Somewhat surprisingly, given the long history of these questions, definitive answers are still largely missing. As with other plant cell walls, one of the central roles of the pollen wall is believed to be protective: during the journey of pollen grains to female structures, the wall shields pollen from various negative factors, such as ultraviolet (UV) radiation, pathogens, and dehydration. Yet, from the purely protective standpoint, the wall patterns are unlikely to matter much. Patterns may, however, be important for other roles that pollen walls play, such as facilitating the efficient delivery of pollen grains to female targets, contributing to pollen hydrodynamics, and mediating interactions between pollen and stigmas, the receptive portion of female structures (Heslop-Harrison, 1971; Muller, 1979; Edlund et al., 2004).

A general correlation exists between pollen surface morphology and plant pollination syndromes; plants pollinated by animals often develop complex patterns with various decorations on their pollen surface, whereas plants pollinated with the help of wind or water tend to have a smoother pollen surface. It has been proposed that various spines, ridges, and papilla on their surface may help pollen grains attach to animal pollinators, while a smooth surface may improve the aerodynamics of pollen (Wodehouse, 1935; Heslop-Harrison, 1971).

The reproductive potential of pollen may also be influenced by surface patterns in several other ways. First, pollen walls often serve as receptacles for the pollen coat – the oily, sticky material that plays a role in interactions with stigmas, pollinators, and other pollen grains (Edlund et al., 2004; Rejón et al., 2016). Differences in pollen surface patterns may affect the total amount of pollen coat that the grain is able to hold, in turn affecting its dispersal and interactions with stigmas. Second, mathematical modelling suggests that differences in wall patterns might influence pollen hydrodynamics, the ability of pollen grains to change volume due to changes in water content without losing viability (Katifori et al., 2010). Third, during the initial adhesion between pollen and stigma, stigmatic cells undergo very rapid morphological changes upon binding pollen of the same species but not of another species, and the exine layer of the pollen wall plays a major role in this process (Zinkl et al., 1999). Although not yet convincingly demonstrated, it is conceivable that pollen patterns may be important for this earliest step of pollen–stigma interaction.

Still, even though examples of correlations between particular pollen patterns and pollination biology have been described (Ferguson and Skvarla, 1982; Osborn et al., 1991; Hesse, 2000; Tanaka et al., 2004; Sannier et al., 2009), the experiments that actually measure the effect of different patterns on pollen dispersal, delivery, and performance are lacking. The discoveries of genes that influence patterning of pollen surface, together with the

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development of efficient gene modification techniques like CRISPR, may soon allow introduction of different pollen patterns into plants beyond the traditional model species. This, in turn, should allow testing of the long-standing hypotheses about the effects of these patterns on pollination and pollen performance.

3 Pollen Wall Structure

Pollen is a male gametophyte, a multicellular (tri- or bicellular in angiosperms) haploid organism in which two tiny sperm cells or their precursor, the generative cell, are encapsulated inside a larger vegetative cell, whose function is to deliver the sperm cells to the egg cell-containing female gametophyte (McCormick, 1993). The vegetative cell is encased in a multilayered pollen wall (Figure 2), sometimes referred to as the *sporoderm*. A typical pollen wall consists of the inner wall, *intine*, located directly above the plasma membrane (PM); the outer wall, *exine*, placed on top of the intine; and the previously mentioned *pollen coat* (also known as *pollenkitt* or *tryphine*) distributed on top of the exine or filling its cavities (Figure 2d). Intine is an unpatterned wall of relatively uniform thickness – although it can be thicker at the aperture sites described in the second half of this article. In its structure and chemical composition, intine is similar to primary cell walls of plant somatic cells – consisting of polymeric carbohydrate materials, like cellulose, pectins, and arabinogalactan proteins (Sitte, 1955; Li et al., 1995). The pollen coat is a sticky, oily substance made of lipids, proteins, aromatic compounds, and pigments (Pacini and Hesse, 2005; Rejón et al., 2016). Neither intine nor pollen coat contributes significantly to the patterns of the pollen surface; these patterns are the product of deposition and assembly of exine.

4 Exine Composition

Among plant cell walls, exine is exceptional in many ways. In addition to its ability to assemble into thousands of patterns of great complexity, it is also made of a material with highly unusual properties. This material, *sporopollenin* (Zetsche, 1932), is remarkably stable and resistant to harsh chemical, physical, microbial, and enzymatic treatments (Southworth, 1974). For example, acetolysis, the method that has been commonly used by palynologists to prepare exine samples for microscopy, involves incubation of pollen at high temperature with concentrated acetic anhydride and sulphuric acid (Erdtman, 1943). This treatment hydrolyses and removes other pollen components (including polysaccharides, proteins, and lipids of intine and pollen coat), leaving sporopollenin-based exine intact and ready for visualisation.

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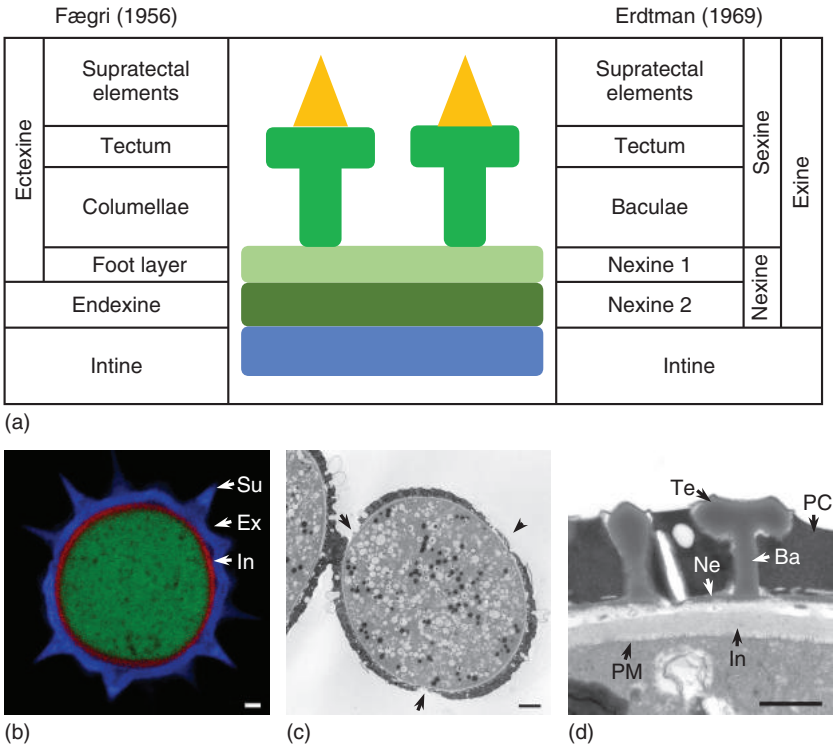

Figure 2 Complex architecture of the pollen wall. (a) Comparison of the two major terminologies used to describe pollen wall components. (b) Confocal image of a sunflower pollen grain. Spectral images of autofluorescence emitted by the grain were captured and spectrally unmixed, demonstrating that different parts of the grain and the wall show differences in their autofluorescence (structures were false-coloured based on their emission spectra: exine – blue; intine – red; cytoplasm – green). Note the presence of spines, the supratectal elements of sunflower exine (Su). (c) Cross section of a mature pollen grain from Arabidopsis, with light-grey cytoplasmic contents surrounded by the darker pollen wall, viewed with the help of transmission electron microscopy (TEM). Arrows indicate three apertures within the exine. (d) Higher magnification TEM image of Arabidopsis pollen shows the details of the pollen wall structure. Ba, Baculum; Ex, Exine; In, Intine; Ne, Nexine; PC, Pollen coat; PM, Plasma membrane; Su, Supratectal elements; Te, Tectum. Scale bars = 2 μm (b, c) and 500 nm (d).

The extraordinary stability of sporopollenin accounts for exine preservation for hundreds of millions of years in fossil material and allows paleobotanists to address questions related to past states of climates and vegetation and determine evolutionary relationships between plants (Crane et al., 1995).

The resistance of sporopollenin to chemical degradation has made it very challenging to establish its composition; although this question has been studied for many years, it is still not definitively resolved (Mackenzie

et al., 2015; Quilichini et al., 2015). Results of the combinations of chemical, spectroscopic and, more recently, genetic approaches indicate that sporopollenin is likely to be a highly cross-linked polymer of hydroxylated fatty acids, aliphatic compounds, and, possibly, phenolics (Guilford et al., 1988; Ahlers et al., 1999, 2000; Domínguez et al., 1999; Wiermann et al., 2005; Morant et al., 2007; Dobritsa et al., 2009b, 2010, 2011; Grienenberger et al., 2010; Kim et al., 2010; Chen et al., 2011; Shi et al., 2011). However, exactly how these putative building blocks are linked together and whether different plant species vary in their sporopollenin composition is poorly understood.

5 Exine Architecture

Despite the enormous variety of different patterns assumed by exines (Figure 1), the overall architecture and stratification of these walls are similar across species. Two major terminologies exist for the description of sub-layers into which exines are organised (Erdtman, 1952, 1969; Fægri, 1956). In this article, we follow the terminology of Erdtman, but the correspondence between the alternative terms is shown in Figure 2a.

Exine is commonly divided into the outer sculptured *sexine* and the simpler inner *nexine*, located just above the intine (Figure 2a,d). The sexine often consists of several structures: the radially directed rods, *baculae* (also called *columellae* because of their column-like appearance); the roof-like *tectum*, supported by the baculae and formed by the fusion of their heads; and the ornamental *supratectal elements* which include various spikes, spines, bumps, globules, and other types of miniature protrusions that develop on the surface of the tectum (Figure 2). The nexine is sometimes subdivided into *nexine I* (or the *foot layer*), and *nexine II* (Figure 2a). Based on their differential staining and differences in electron density in micrographs, the sub-layers of nexine may have different compositions. In different species, exines may lack certain layers or structures: pollen grains of many species do not develop supratectal elements, tectum, or nexine substructures. Depending on the way exine assembles, patterns visible on the surface, without sectioning the pollen grain, might be created by supratectal elements, tectum, baculae, or by various combinations of exine's elements and layers.

6 Exine Ontogeny

The general outline of events happening during exine formation is shown in Figure 3 and is well described (Echlin and Godwin, 1968a; Heslop-Harrison, 1968a, 1971; Scott, 1994; Owen and Makaroff, 1995; Blackmore et al., 2007; Wilson and Zhang, 2009; Ariizumi and Toriyama, 2011; Lou et al., 2014;

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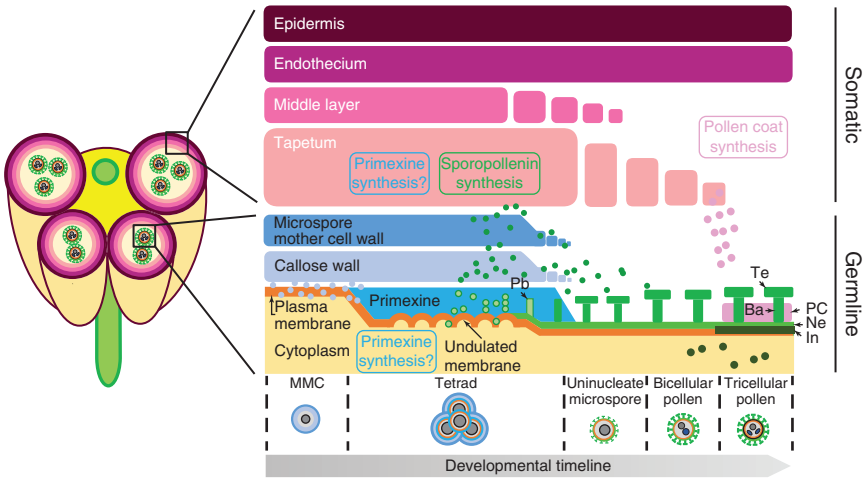

Figure 3 Anther structures and developmental events associated with formation of the pollen wall. Multiple walls are built and destroyed around the germline cells prior to the formation of mature exine. Ba, Baculae; In, Intine; MMC, Microspore mother cell; Ne, Nexine; Pb, Probaculae; PC, Pollen coat; Te, Tectum.

Quilichini et al., 2014a; Shi et al., 2015). The first signs of exine formation appear at the tetrad stage of pollen development when four microspores, the haploid products of meiotic division, are transiently kept together. Prior to entering meiosis, microspore mother cells (MMCs), the diploid precursors of pollen, surround themselves with a temporary wall made of polysaccharide callose (β-1-3-glucan). This wall, which develops underneath the original primary cell wall of the MMC, physically isolates MMCs from the neighbouring sporophytic layers and, after meiosis, allows microspores to be transiently kept as tetrads. Later, upon degradation of the callose wall, the individual microspores are released into the anther locule where they continue their transformation into mature pollen grains; the construction of the pollen wall becomes one of the most obvious hallmarks of this transformation.

During the tetrad stage, each of the microspores develops a thin layer of *primexine* between its PM and the callose wall (Figure 3). Primexine is an ephemeral, poorly defined fibrillar matrix that precedes exine formation and is generally believed to be involved in exine patterning (Ariizumi and Toriyama, 2011; Lou et al., 2014; Shi et al., 2015). It is sometimes referred to as 'exine template' (Skvarla and Larson, 1966; Skvarla and Rowley, 1987; Takahashi, 1987) because sporopollenin (or its precursors) accumulates within the primexine matrix at distinct positions. Whether primexine indeed provides pre-patterning instructions for exine, and itself has any obvious patterning, is still unclear. The fact that primexine is short-lived and has low electron density makes it challenging to visualise and characterise it.

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Unlike exine, with its unusual sporopollenin-rich composition, primexine is believed to be similar in chemical structure to the primary cell walls of plants, composed of carbohydrate-based polymers, like pectins and cellulose (Heslop-Harrison, 1968b; Rhee and Somerville, 1998).

Around the time of primexine formation, the PM of each microspore in a tetrad starts separating from the callose wall, forming a regular pattern of invaginations (usually referred to as the '*plasma membrane undulation*') (Figure 3). Several studies of exine formation in Brassicaceae in which samples were prepared using cryo-fixation also reported the appearance of '*spacers*', the thickest and most electron dense regions of primexine that are deposited with regularity between the peaks of the undulating membrane (Fitzgerald and Knox, 1995; Paxson-Sowders et al., 2001; Quilichini et al., 2014a). PM undulation has been suggested to be the first manifestation of the future patterning; it is assumed to guide the eventual distribution of baculae, with the precursors of baculae (*probaculae*) developing specifically at the peaks of the undulating membrane (Paxson-Sowders et al., 2001; Quilichini et al., 2014a). The height of primexine may regulate the height of probaculae (Takahashi and Kouchi, 1988).

The order of the events that follow may differ between taxa: in some species (e.g. *Bougainvillea spectabilis* and *Lilium longiflorum*), formation of first protectum and then probaculae has been described (Takahashi and Skvarla, 1991; Takahashi, 1995a); in others (e.g. *Hibiscus* sp.), probaculae are apparently formed before the protectum (Takahashi and Kouchi, 1988). The nexine layer is typically the last to develop. By the time, the callose wall is degraded and the free microspores are released, the thin patterned exine is already present on their surfaces (Figure 3). During the free microspore stage, exine grows significantly in thickness and electron density, due to continuous sporopollenin deposition.

The timing of formation of supratectal elements might differ between species. For example, spines in *Hibiscus* (Malvaceae) (Figure 1e) were found to develop after dissolution of the callose wall, at the free microspore stage (Takahashi and Kouchi, 1988; Takahashi, 1994). In contrast, in some species of Compositae, the protectum already assumes the spiky echinate pattern during the tetrad stage (Takahashi, 1989). Also, in *Nuphar* (Nymphaceae) elongating spines were found to appear in the callose wall at the tetrad stage before the formation of any other exine component (Takahashi, 1992).

7 Sporophytic Control of Exine Formation: Microspore Mother Cells and Tapetum

One of the most unusual things about exine is the way its development is controlled. Although exine develops around haploid gametophytes and the

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first signs of its formation do not appear until the haploid stage of pollen development is reached, the control of exine deposition and patterning is provided by the diploid genome of a sporophyte, as evidenced by the genetic analysis of multiple available exine mutants (Paxson-Sowders et al., 2001; Ariizumi et al., 2004; Morant et al., 2007; Yang et al., 2007; Suzuki et al., 2008; Dobritsa et al., 2009a,b, 2010; Grienenberger et al., 2010; Kim et al., 2010; Quilichini et al., 2010; Chang et al., 2012; Li et al., 2017; Suzuki et al., 2017). Moreover, at least two classes of sporophytic cells, MMCs and tapetum, appear to contribute to exine formation. MMCs are possibly responsible for the exine patterning information. Centrifugation experiments performed on lily anthers at different stages of microsporogenesis suggested that determinants of pollen patterns are already present in MMCs prior to meiosis, as patterns of exine became disrupted in some cells that were centrifuged at that time (Sheldon and Dickinson, 1983). In addition, enucleated cells and cells treated with colchicine retain an ability to develop quite regularly patterned exine (Heslop-Harrison, 1971; Sheldon and Dickinson, 1983, 1986), suggesting that the synthesis of new patterning determinants after meiosis/cytokinesis is not required and that positioning of meiotic spindle also does not play a role in exine patterning.

Unusually for a plant cell wall, the majority of sporopollenin precursors of exine are produced not by the sporogenic cells that develop into pollen but by the nearby *tapetum*, the innermost cell layer of the anther wall surrounding the locule where pollen develops (Figure 3). The sporopollenin precursors are then secreted from the tapetum into the anther locule and transported to the surface of the developing free microspores where they assemble into species-specific patterns preprogrammed during the tetrad stage (Ariizumi and Toriyama, 2011; Quilichini et al., 2014a, 2015). Exactly how the transport of various factors from tapetum to microspores is organised is still poorly understood. In some species, like rice (*Oryza sativa*), sporopollenin precursors appear to be delivered from the tapetum in the form of small (close to one micrometer or less) spherical structures, known as *Ubisch bodies* or *orbicules* (Echlin and Godwin, 1968b; Huysmans et al., 1998). The surface patterns of orbicules are often strikingly similar to patterns on the pollen surfaces of the same species, and they become modified in mutants in which exine formation is affected (Huysmans et al., 1998; Liu et al., 2017). While distinct orbicules are not visible in the wild-type Arabidopsis and other Brassicaceae, accumulation of some orbicule-like structures in anther locules was noticed in Arabidopsis mutants in which tapetal transport was disrupted (Quilichini et al., 2014b). Yet, how orbicules or other tapetal materials required for exine formation are directionally transported through the anther locule to the microspore surface remains unknown.

Although it is well known that tapetum is responsible for the biosynthesis of sporopollenin building blocks in the post-tetrad period, it is less clear where the materials contributing to the formation of primexine and other

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early structures, like probaculae and protectum, come from. It is often assumed that the callose wall surrounding the tetrad-stage microspores is impenetrable to macromolecules and even to smaller molecules, like thymidine (Heslop-Harrison and Mackenzie, 1967; Knox and Heslop-Harrison, 1970); in this case, it would be reasonable to accept MMCs or microspores as the source of the exine precursor materials in the tetrad period. However, some studies questioned the callose wall's ability to act as an impenetrable barrier, as it showed permeability to colloidal iron, alkaloid colchicine, and heavy cerium ions (Rowley and Dunbar, 1970; Heslop-Harrison, 1971; Rodriguez-García and Majewska-Sawka, 1992). This brings up a possibility that tapetum may contribute to exine formation not only during the free microspore stage, but also earlier, in the tetrad stage when the patterning of exine is initiated and may, therefore, act as the sporophytic source of at least some patterning information.

8 Molecular Mechanisms of Exine Patterning: Insights from Mutant Analysis

Even though the main events taking place during exine formation have been known since the 1960s, there are still many unsolved puzzles and mysteries in this process. Among those are the exact roles of the callose wall, primexine, and PM undulation in the formation of exine patterns, as well as the origin of patterning information. The identification of a large number of exine mutants and gene candidates in recent years, primarily in Arabidopsis and rice, led to significant progress towards characterising the molecular processes guiding exine formation, particularly in regard to the biosynthesis of exine precursors in tapetum, their transport to the microspore surface, and the transcriptional regulation of these processes. Several excellent recent reviews describe these processes (Lou et al., 2014; Gómez et al., 2015; Quilichini et al., 2015; Shi et al., 2015). In this article, we instead focus on the select genes and mutants that could provide information about exine patterning.

8.1 Formation and Degradation of the Callose Wall

Mutant analysis in Arabidopsis has shown that the callose wall plays an essential role in the development of exine patterns. Mutations in the *CALLOSE SYNTHASE5* gene (*CALS5*, also known as *GLUCAN SYNTHASE-LIKE2* (*GSL2*)), which lead to the lack or abnormal formation of the callose wall around MMCs result in the development of exine that no longer assembles into the reticulate pattern characteristic of Arabidopsis, but instead consists of globules of sporopollenin randomly distributed on the pollen surface (Dong et al., 2005; Nishikawa et al., 2005). Similarly, other Arabidopsis mutants that

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exhibit defects in callose deposition (like the ones affecting genes for the sugar transporter RUPTURED POLLEN GRAIN1 (RPG1), CYCLIN-DEPENDENT KINASE G1 (CDKG1), AUXIN RESPONSE FACTOR17 (ARF17), and the rhomboid-type protease KOMPEITO) develop similar defects in their exine patterns (Guan et al., 2008; Thompson et al., 2012; Huang et al., 2013; Sun et al., 2013; Yang et al., 2013). These findings led to the idea that during exine development the callose wall provides a platform against which the tectum forms. Consistent with this idea, in some aquatic plants (e.g. *Ceratophyllum*) in which the callose wall is either not synthesised or its synthesis is greatly reduced, pollen develops structureless, unsculptured exine that lacks components of sexine (Pettitt, 1981; Takahashi, 1995b).

The timing of callose wall degradation also appears to be critical for exine formation; when the callose wall in tobacco was dissolved prematurely during microsporogenesis (by expressing a pathogenesis-related callase/β-1,3-glucanase from the tapetum-specific *A9* promoter), this led to disrupted exine sculpturing, with the resulting malformations of pollen wall interpreted as the failure of tectum to develop (Worrall et al., 1992).

The callose wall is believed to be degraded by tapetally secreted callase activity (Stieglitz, 1977). In Arabidopsis, 18 putative β-1,3-glucanases are expressed in buds at the time of microspore release, with six genes exhibiting high levels and high specificity of expression in tetrad-stage anthers (Tratt, 2016). However, it is unclear which of these putative glucanases are involved in the degradation of the callose wall, as analysis of single and multiple knockout mutants for these genes (up to quadruple for the strongest candidates) did not reveal any abnormalities in callose degradation – possibly due to a high degree of functional redundancy between the genes (Tratt, 2016). Alternatively, genes identified as the best candidates for involvement in tetrad callose degradation may in fact play other roles; in agreement with this, premature expression of the strongest candidates for the role of tetrad callase, the *AtA6* gene (At4g14080) (Hird et al., 1993), and its close homologue *AtA6F* (At3g23770), from the *CALS5* or *A9* promoters failed to produce any obvious effect on the callose walls surrounding tetrads (Tratt, 2016).

In rice, however, a possible tetrad callase has been found. The *Osg1* gene is expressed in anthers during microspore release and encodes a β-1,3-glucanase with high similarity to pathogenesis-related β-1,3-glucanases (Wan et al., 2011). Importantly, the suppression of Osg1 activity by RNAi led to the disruption of callose wall degradation and delayed release of microspores, suggesting that Osg1 may be a *bona fide* tetrad callase (Wan et al., 2011).

8.2 Primexine Formation and Plasma Membrane Undulation

Although the exact roles played by primexine and PM undulation in exine formation are not yet understood, it is widely assumed that they must be

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connected to exine patterning (Blackmore et al., 2007; Ariizumi and Toriyama, 2011; Lou et al., 2014; Shi et al., 2015). Several mutants with defects in primexine formation and PM undulation have been found in Arabidopsis and rice, and all exhibit abnormalities in regular sporopollenin attachment and baculae formation; as mature plants, most of these mutants show male sterility due to pollen degeneration (Paxson-Sowders et al., 1997, 2001; Ariizumi et al., 2004; Guan et al., 2008; Chang et al., 2012; Hu et al., 2014). Most of the genes defective in these mutants encode proteins whose function is not immediately clear, and few, if any, follow-up studies on these genes have been performed after the initial reports. Thus, although several molecular players required for primexine formation or PM undulation have been identified, they have not so far significantly clarified how these processes are related to exine development.

DEFECTIVE EXINE1 (*DEX1*) encodes a novel membrane protein with some similarity to calcium-binding domains of animal integrins, the transmembrane proteins that facilitate interactions between intracellular cytoskeleton and components of extracellular matrix (Paxson-Sowders et al., 2001). In the tetrad-stage *dex1* mutants, multiple abnormalities were observed at the PM–callose wall interface: delayed and abnormal deposition of primexine, lack of PM undulation, loss of normal probaculae development, and very broad electron-dense deposits appearing on the microspore surface (Paxson-Sowders et al., 1997, 2001). A combination of these defects and the similarity to integrins led to hypotheses that DEX1 may reside in the microspore PM and act either as a component of primexine, a factor required for primexine assembly, a protein that mediates contacts between the peaks of undulating PM and the callose wall, or a receptor for sporopollenin (Paxson-Sowders et al., 1997, 2001). A later study, however, found that DEX1 is abundantly expressed in the tapetal layer, leading to suggestions that it may be involved in the transport of exine precursors from tapetum rather than (or in addition to) their reception by microspores (Ma et al., 2013). A recent study of the DEX1 homologue in rice, OsDEX1, found that it is similarly required for primexine and exine formation in this species and drew attention to the protein's calcium-binding abilities, suggesting that OsDEX1 might be involved in regulating calcium homeostasis in tapetum (Yu et al., 2016).

Several other Arabidopsis genes have been shown to be required for primexine formation and PM undulation. Like *DEX1*, the aptly named *NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION* (*NPU*) also encodes a novel protein. This protein has two predicted transmembrane domains, is expressed in both tapetum and MMCs, and is expected to localise to the PM (Chang et al., 2012). *NO EXINE FORMATION1* encodes another novel protein with some limited similarity to membrane proteins or transporter-like proteins (Ariizumi et al., 2004). RPG1 (also known as SWEET8) and its homologue RPG2 are members of the MtN3/saliva family

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of transmembrane proteins that act as sugar transporters (Guan et al., 2008; Sun et al., 2013). Mutations in *EXINE FORMATION DEFECT* (*EFD*) result in the formation of a very thin primexine and the loss of probaculae. *EFD* encodes a *de novo* methyltransferase that is strongly expressed both in tapetum and MMCs, and its mutations correlate with downregulation of multiple genes required for exine formation, including *RPG1* (Hu et al., 2014). Mutations in *ARF17* (mentioned earlier in relation to callose wall synthesis) also cause apparent lack of primexine deposition and PM undulation (Yang et al., 2013). Interestingly, mutations in all of the above-mentioned primexine-related genes also affect *CALS5* gene expression and callose wall formation (Guan et al., 2008; Chang et al., 2012; Ma et al., 2013; Sun et al., 2013; Yang et al., 2013; Hu et al., 2014), although the defects observed during microsporogenesis and exine formation in these mutants typically stretch beyond those associated with *cals5* mutations.

8.3 Genes Associated with Carbohydrate-Related Processes

Although multiple genes required for exine formation were discovered through analysis of male-sterile mutants whose pollen does not survive to maturity, two forward genetic screens in Arabidopsis have demonstrated that mutants with defective exine patterning can also be found through visual screening of viable pollen (Suzuki et al., 2008; Dobritsa et al., 2011). These screens identified a number of mutants in which exine was formed but developed multiple abnormalities, leading to the loss of regular reticulate structure: those included denser distribution of baculae, loss of tectum or, instead, an overdeveloped tectum with smaller lacunae, missing tectal connections, and variations in patterns between different areas on the pollen surface. Interestingly, several genes discovered via these screens encode proteins related to carbohydrate biosynthesis.

The gene *UNEQUAL PATTERN OF EXINE1/KAONASHI4* (*UPEX1/KNS4*) encodes a β-1,3-galactosyltransferase that likely participates in the biosynthesis of arabinogalactan type II core of arabinogalactan proteins (AGPs) or of rhamnogalacturonan I (Dobritsa et al., 2011; Suzuki et al., 2017). The *upex1/kns4* mutants develop very short baculae and patchy exine, often with small lacunae. The genes *SPONGY2/IRREGULAR XYLEM9-LIKE* (*SPG2/IRX9L*) and its homologue *IRX14L* encode putative xylosyl transferases involved in the biosynthesis of the polysaccharide xylan (Dobritsa et al., 2011; Li et al., 2017). Analyses of the *upex1/kns4* and *spg2/irx9l* mutants, coupled with antibody staining for several carbohydrates during microsporogenesis, revealed defects in primexine formation and led to suggestions that AGPs, pectins, and xylan are likely constituents of primexine (Li et al., 2017; Suzuki et al., 2017).

Interestingly, confocal microscopy was able to detect patterns formed by low-methylesterified pectins, and AGPs on the surfaces of microspores at

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the late tetrad/early free-microspore stage (Suzuki et al., 2017). AGPs were initially assembled in patterns coinciding with the reticulate arrangement of developing exine. The AGP signal was distributed at the top of the putative primexine layer and appeared somewhat later in development compared to pectins. At later stages AGPs became localised to exine cavities. In contrast, pectins around microspores appeared earlier than AGPs, localised to the bottom of the primexine layer, and were confined to the cavities of developing exine throughout the course of its development (Suzuki et al., 2017).

Based on these findings, Suzuki et al. (2017) proposed a model for the role of AGPs and pectins in construction of exine. According to this model, microspores at the late tetrad stage are surrounded by primexine mainly composed of pectin, providing the initial matrix into which sporopollenin is deposited. Upon deposition of AGPs on top of primexine, the tectum of the fledgling exine becomes sandwiched between AGPs and pectins. Later, continued deposition of AGPs into primexine helps lift baculae of exine to their proper height, while continuous deposition of sporopollenin confines AGPs to exine cavities. Interestingly, *UPEX1/KNS4* is specifically expressed in tapetum (Li et al., 2017; Suzuki et al., 2017), providing support for the idea that tapetal components may be involved in exine patterning.

The *KAONASHI2* (*KNS2*) gene also encodes an enzyme linked to carbohydrate synthesis – a sucrose phosphate synthase, which catalyses sucrose biosynthesis from uridine diphosphate (UDP)–glucose and fructose-6-phosphate (Suzuki et al., 2008). The defects in *KNS2* lead to higher density of baculae and development of smaller lacunae in the tectum. It remains to be seen, however, if some aspects of primexine formation are affected in this mutant.

9 Physicochemical Models of Exine Patterning

In addition to biological explanations for exine patterning, there are also models that propose that polymerising sporopollenin spontaneously organises into specific patterns, guided by principles of physical chemistry (Heslop-Harrison, 1972; van Uffelen, 1991; Gabarayeva, 1993; Hemsley and Griffiths, 2000; Gabarayeva and Hemsley, 2006; Hemsley and Gabarayeva, 2007). The biological and physicochemical explanations are not necessarily mutually exclusive, as biological mechanisms are expected to specify conditions for sporopollenin self-assembly. Interspecific differences in these conditions (e.g. in factors like elasticity and tension of the membrane, composition and density of proteins, lipids, and carbohydrates in the PM, callose wall, and primexine, osmolarity of the locular fluid, and the exact chemistry of sporopollenin precursors) could potentially account for the diversity of patterns.

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A phenomenological biophysical model of first-order patterning transitions on a sphere, tailored to pollen grains, has been recently developed (Lavrentovich et al., 2016). According to this model, the phase separation of compounds with different chemical properties, such as carbohydrates in primexine and callose wall and hydrophobic precursors of sporopollenin, coupled with the irregularities of PM undulation, can drive pattern formation on the surface of the microspore PM. The initiation of patterning may occur at a localised site, but then becomes reiterated and spreads to the entire surface. The model also suggests that the spherical topology of pollen grains might be responsible for the enormous diversity of exine patterns: compared to flat, planar surfaces, spheres introduce topological defects expected to yield rich patterning variations (Lavrentovich et al., 2016). Experiments with colloids and surfactants in various water–lipid mixtures show that formation of aggregates resembling patterns of various exines is indeed possible (Hemsley et al., 2003; Hemsley and Gabarayeva, 2007; Gabarayeva and Grigorjeva, 2016).

10 Pollen Apertures: Patterns and Functions

In addition to the patterns formed by the assembly of exine, pollen surfaces almost always exhibit patterns formed by the gaps in exine deposition (Figures 1, 2c and 4a). These areas of reduced exine are known as *pollen apertures*. Like other patterns on the pollen surface, aperture patterns are usually species specific and exhibit large interspecies variations; apertures can differ in shape (e.g. elongated furrows, pores, or a combination of these types), in number (from zero to many), in size, positions, orientations, ornamentation, and margin characteristics (Wodehouse, 1935; Walker and Doyle, 1975; Furness and Rudall, 2004; PalDat, 2017). The two most common prototypical aperture patterns are a single polar aperture, present in pollen of multiple monocots, and three apertures located at the pollen equator, found in the majority of eudicots (Walker and Doyle, 1975; Blackmore and Crane, 1998; Furness and Rudall, 2004). However, both phylogenetic clades also include many species whose aperture patterns deviate significantly from these prototypes (Blackmore and Crane, 1998; Nadot et al., 2006; Matamoro-Vidal et al., 2016; PalDat, 2017).

As the surface elements disrupting exine continuity, apertures have been suggested to play multiple roles in communication between pollen and its environment (Wodehouse, 1935; Heslop-Harrison, 1976, 1979a; Muller, 1979; Edlund et al., 2004). Most commonly, apertures are thought of as weaker areas, providing sites through which pollen tubes can germinate. A recent study done with the help of atomic force microscopy estimated that aperture regions (covered only by intine and, in some cases, nexine) could be an order

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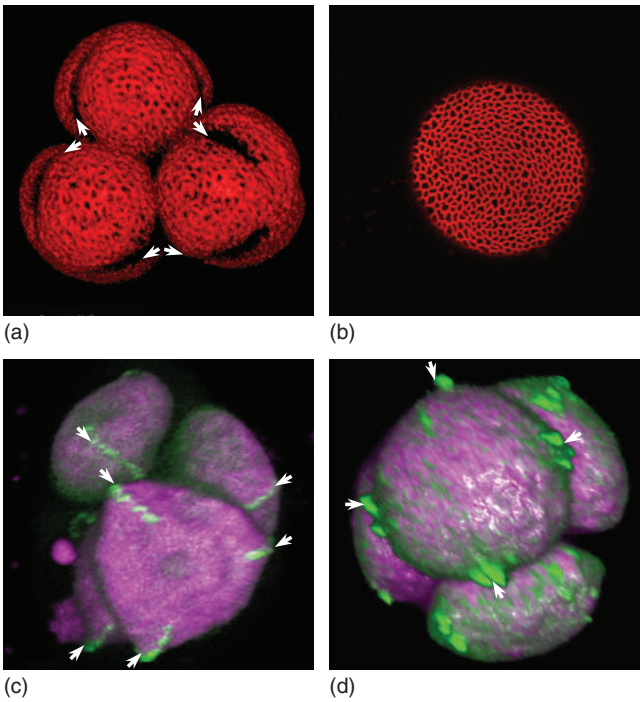

Figure 4 Pollen apertures as a model for formation of distinct cellular domains. (a) Each pollen grain in Arabidopsis develops three equidistant longitudinal apertures, which at the tetrad stage are aligned with apertures on sister microspores. Shown is a late-stage Arabidopsis tetrad (three out of four microspores are visible in this view); each of the two apertures visible in each of the three microspores is aligned with an aperture on the sister microspore, creating a total of six aperture-pairs per tetrad (arrows point to pairs of apertures). (b) Apertures are completely lost in the *inp1* mutant of Arabidopsis. (c) INP1 protein (green) assembles at three membrane domains in each tetrad-stage microspore, forming three longitudinal punctate lines per microspore (arrows). Microspores are stained with a membrane dye (magenta). (d) When ploidy of microspores is increased, INP1 localises to more membrane domains (four in the case of diploid microspores shown here, arrows).

of magnitude weaker than the regions covered by regular exine (Edlund et al., 2016). In many species, pollen tubes indeed appear to exit preferentially or exclusively through the apertures (Heslop-Harrison, 1979b; Heslop-Harrison and Heslop-Harrison, 1985; Edlund et al., 2016). In the pollen of maize, for example, tubes seem to emerge only through their single pore-like apertures (Li et al., 2018). The crucial role of apertures in maize (and most likely in other grasses as they all share very similar exine and aperture patterns (Zavada, 1983; Linder and Ferguson, 1985)) was confirmed by the recent discovery of the *zminp1* mutant. This maize mutant in which pollen lacks apertures

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(inaperturate phenotype) fails to germinate pollen tubes and shows complete male sterility (Li et al., 2018).

However, not all species exhibit such exclusive reliance on apertures for pollen tube emergence. The pollen of the Arabidopsis mutant defective in *INAPERTURATE POLLEN1* (*INP1*), the homologue of the maize *ZmINP1*, similarly loses its apertures (Figure 4b), yet exhibits normal fertility (Dobritsa et al., 2011). It was recently shown that in Arabidopsis and some other Brassicaceae species pollen tubes often emerge through exine, preferring to take the shortest route into the stigma even when three normal apertures, typical of this family, are present in the pollen (Edlund et al., 2016). This 'breaking-through-the-wall' phenomenon may be due to the specific morphology of exine in the pollen of Arabidopsis and some of its relatives (e.g. in contrast to the thicker exine with fully covered tectum in maize, exine in Arabidopsis is thinner and has mesh-like (reticulate) tectum), and also, possibly, due to physiology of pollen and stigma in these species. It was recently proposed that, in these species, the presence of hydrogen peroxide, catalase, peroxidase, and possibly some other reactive oxygen species (ROS)-related factors at the pollen–stigma interface may oxidise sporopollenin, weakening exine, and allowing pollen tubes to escape through the interapertural regions (Edlund et al., 2017). In addition, some species normally produce inaperturate pollen (PalDat, 2017) – although this characteristic is uncommon and is often associated with other unusual traits (e.g. thin exine, aquatic lifestyle, and functionally female flowers) that may reduce pollen dependence on apertures for germination (Furness, 2007).

In addition to commonly providing portals for pollen tube germination, apertures also frequently serve as the sites through which water enters and exits pollen (Heslop-Harrison, 1979c; Vieira and Feijó, 2016), as well as the architectural details that help pollen accommodate volume changes in response to changing hydration conditions (Wodehouse, 1935; Edlund et al., 2004; Halbritter and Hesse, 2004; Katifori et al., 2010). A pollen grain typically undergoes several rounds of shrinkage and expansion, most notably losing its volume when exposed to air upon release from anthers and absorbing water and increasing its size upon contact with stigma (Heslop-Harrison, 1979c; Firon et al., 2012). At such moments, presence of apertures may facilitate stress redistribution and shape changes, allowing pollen covered with the relatively rigid exine to expand and contract without breaking. To prevent excessive water loss in dry environments, pollen with elongated apertures folds inwards at the aperture sites (a process termed *harmomegathy*), effectively sealing the inside of the pollen grains to maintain viability (Wodehouse, 1935; Blackmore and Barnes, 1986; Katifori et al., 2010). Mathematical modelling of pollen deformations suggested that aperture patterns – in particular, aperture shape – likely play a critical role in ensuring predictable and reversible pollen folding: with apertures in the shape of

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elongated furrows providing the conformation that is the most energetically favourable for folding (Katifori et al., 2010). This may explain the prevalence of furrow-shaped apertures (colpi and sulci) among angiosperms (Furness and Rudall, 2004).

11 The Role of Aperture Patterns in Pollen Performance

Like the diversity of micropatterns of exine, the diversity of aperture patterns across species has been arousing interest for a long time, leading to hypotheses about their physiological significance and the driving forces that help maintain certain kinds of patterns (Tammes, 1930; Heslop-Harrison, 1979c; Edlund et al., 2004; Furness and Rudall, 2004; Furness, 2007; Matamoro-Vidal et al., 2016).

In the evolution of flowering plants, trends towards a higher number of apertures appeared several times, suggesting that an increased number of apertures may be functionally significant (Furness and Rudall, 2004). It has been previously proposed that selection may favour an increased number of apertures because it improves the chances of contact between the areas on the pollen surface adapted for water transfer and the stigmatic cells, which serve as the source of water for pollen hydration, especially in species with dry stigmas (Heslop-Harrison, 1979c). Heslop-Harrison suggested that the forate aperture pattern consisting of multiple circular apertures (also known as pantoporate pattern) (Figure 1e,h), found exclusively in species with dry stigmas, might have arisen as such an adaptation. Pollen with this pattern tends to undergo hydration via the aperture closest to the stigmatic surface, and this is followed by the highly localised pollen tube emergence through the same aperture (Heslop-Harrison et al., 1975; Heslop-Harrison, 1979c). When a single small aperture occupies a tiny portion of the pollen surface (e.g. in grasses (Figure 1f)), additional paths for water transfer – like microchannels in the nexine – must be present (Heslop-Harrison, 1979c).

In addition to providing a conduit for water that allows pollen germination, apertures also present a certain liability, as the primary sites through which water is lost when pollen undergoes dehydration. Thus, aperture patterns likely represent successful compromises that evolved in response to competing selective pressures to ensure pollen survival and reproductive success. One example of aperture pattern modification that may have an adaptive role is the formation of *opercula* (Figure 1f). These exinous structures develop above apertures in some species (Hesse et al., 2009; PalDat, 2017) and act as lids that open and close; opercula can fit into the apertures during the dehydration phase, sealing the grains and preventing further water loss, and can be lifted during the rehydration phase to allow pollen hydration and pollen tube germination. Consistent with their adaptive role, opercula

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are frequently present in the pollen of species living in dry environments and are notably absent in the species growing in moist habitats (Furness and Rudall, 2003).

12 Triaperturate Pattern: A Key to the Success of Eudicots?

As mentioned above, eudicot species most commonly produce pollen with three equidistant apertures. The tricolpate or, more generally, the triaperturate pollen pattern is considered to be one of the few synapomorphies of the eudicot clade (Donoghue and Doyle, 1989; Judd and Olmstead, 2004). The prevalence of this aperture pattern among eudicots, which themselves constitute approximately three quarters of modern angiosperm species, led to suggestions that this pattern might represent the optimal adaptation for pollen performance (Furness and Rudall, 2004; Matamoro-Vidal et al., 2016).

However, measuring the effects of aperture patterns on pollen performance has been notoriously difficult. Until recently, very few such experiments have been attempted, and those that have been performed included set-ups that were far from ideal, either comparing pollen from different species that have different aperture patterns (potentially introducing other variables besides aperture patterns), or comparing common and rare pollen aperture morphs produced by the same plant (with the complication that rare morphs are hard to identify) (Dajoz et al., 1991, 1993; Till-Bottraud et al., 1999). The recently introduced Arabidopsis pollen aperture series, comprising plants of the same species that produce pollen with either zero, three, four, or four-to-six apertures, overcame some of the drawbacks of the previous studies and provided an alternative way to measure the effects of aperture number on pollen longevity, response to osmotic stress, germination, and reproductive success (Prieu et al., 2016; Albert et al., 2018). These experiments suggest that although inaperturate pollen exhibited the highest resistance to osmotic stresses, the tricolpate pollen indeed appeared to be the strongest overall performer across the panel of assays, consistent with the idea that this pattern may represent the best trade-off. Some caution, however, must be exercised when interpreting these results. Although this experimental set-up is probably the best available at the moment for these types of questions, it still suffers from certain caveats – such as the higher ploidy of the pollen with more than three apertures (Reeder et al., 2016; Albert et al., 2018), and the relatively unusual ability of Arabidopsis pollen tubes to exit through exine in the interapertural areas (Edlund et al., 2004, 2016).

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13 Pollen Apertures as a Model for Generation of Cell Polarity and Formation of Distinct Membrane and Extracellular Domains

Because of their highly stereotypical development within a species, apertures could be used as a valuable model to study the processes of generation of cell polarity and formation of distinct cellular and extracellular domains (Reeder et al., 2016; Dobritsa and Reeder, 2017; Dobritsa et al., 2018). For example, pollen of the wild-type Arabidopsis always develops three narrow longitudinal apertures, equidistantly placed around the pollen equator (Figure 4a) (Bronkers, 1963; Dobritsa et al., 2011). This indicates that three equidistant areas on the surface of developing pollen are reliably specified as future aperture regions, suggesting that these areas provide some signals to the exine deposition machinery that prevent exine development at these sites.

Like with exine formation, the first signs of apertures appear after meiosis, when tetrad-stage microspores are held together by the common callose wall. During the time when the PM initiates separation from the callose wall, forming characteristic membrane undulations, and the primexine is deposited between the PM and the callose wall, certain PM regions remain in close contact with the callose wall and fail to undergo primexine deposition (Echlin and Godwin, 1968a; Heslop-Harrison, 1968b; Dobritsa et al., 2018). Presumably, this in turn prevents formation of exine at these regions and eventually leads to the development of apertures. What makes the PM at the future-aperture regions remain in close proximity to the callose wall and how these particular regions are selected and specified, so that a defined number of apertures with specific morphology will develop at certain positions, are intriguing and poorly understood questions.

To identify genes involved in aperture formation, forward genetic approaches can be used. The great variability of aperture patterns in nature suggests that the system is highly malleable and that genetic screens are likely to identify mutants with variations in aperture patterns. In species like maize and rice, in which apertures seem to have a strong impact on pollen fertility (Li et al., 2018), aperture mutants may be found in screens focused on male sterility. In Arabidopsis, which is much more tolerant to aperture loss and modifications, simple visual screens can be successful in isolating aperture mutants (Dobritsa et al., 2011; M. Tan, P. Amom, and A. A. Dobritsa, unpublished). Variations in aperture patterns in Arabidopsis are visible with the help of low-magnification microscopy as changes in the shape of dry pollen grains (Dobritsa et al., 2011, 2018; Reeder et al., 2016). This facilitates visual screens that do not require lengthy sample preparations and can be easily performed with minimal training. Depending on the exact modifications of aperture patterns, the characteristic oval shape of the wild-type triaperturate Arabidopsis pollen can change into a round, square,

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or triangular shape in mutants. Follow-up studies with confocal microscopy allow viewing of apertures on the entire pollen surface to establish their numbers and positions and recognise changes in their patterns (Dobritsa et al., 2011, 2018; Reeder et al., 2016).

A forward genetic screen in Arabidopsis resulted in the isolation of the above-mentioned *INP1* gene, the first discovered aperture-specific molecular factor whose inactivation leads to the complete loss of apertures (Figure 4b) (Dobritsa et al., 2011; Dobritsa and Coerper, 2012). Studies of this gene have provided interesting insights into the formation of apertures and of the corresponding PM domains. *INP1* encodes a novel protein with no recognisable domains of known function but with a very distinct and unusual pattern of cellular localisation. In the developing wild-type tetrad-stage microspores, INP1 localises to the three narrow domains of PM that will eventually develop into apertures, forming longitudinal punctate lines along these domains (Figure 4c) (Dobritsa and Coerper, 2012; Dobritsa et al., 2018). Interestingly, in mutants with an abnormal number of apertures (e.g. four or six instead of three), INP1 changes its positions accordingly (Figure 4d), assembling into the corresponding number of punctate lines (Reeder et al., 2016; Dobritsa et al., 2018). Aperture length appears to be sensitive to the INP1 levels, as plants with reduced INP1 expression form shorter than normal apertures (Dobritsa and Coerper, 2012). This suggests a possibility that the presence of brevicolpate apertures (short furrows) in plant families which also include species with long apertures may be caused by mutations that modulate expression levels of INP1 or other aperture factors.

However, not all aspects of aperture morphology appear to be controlled by INP1, as indicated by the experiments in which the lack of INP1 function in the Arabidopsis *inp1* mutant was complemented by expression of CrINP1, an INP1 homologue from another member of Brassicaceae, *Capsella rubella* (Li et al., 2018). This species has apertures that are wider than the ones in Arabidopsis and have irregular margins and internal exine deposits, absent in Arabidopsis. The apertures that were restored by CrINP1 had an Arabidopsis-like, and not a Capsella-like, morphology.

The involvement of INP1 in aperture formation appears to be conserved in evolution, since it acts as an aperture factor not only in Brassicaceae, but also in such distantly related species as maize (Li et al., 2018). This finding is particularly interesting because both the aperture patterns and the INP1 protein sequences are very different between grasses and Brassicaceae. In order to perform their functions, INP1 proteins possibly require species-specific partners, since the INP1 homologues from tomato, California poppy, and the grass Brachypodium (the latter being very similar to maize in its INP1 sequence and aperture patterns) were unable to complement the defects in the Arabidopsis *inp1* mutant, despite the likely involvement of these genes in the process of aperture formation in their respective species (Li et al., 2018).

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What brings INP1 to the PM aperture domains and what keeps it there is completely unknown at the moment. The INP1 protein structure provides few clues as to how the protein is kept at the PM, although recent experiments involving Arabidopsis-tomato INP1 chimeras suggest that the central portion of INP1 might mediate its interactions with species-specific partners (Li et al., 2018). Several lines of evidence suggest that, while INP1 is absolutely essential for aperture formation, it is unlikely to act early in aperture development to specify aperture domains: (i) The higher levels of INP1 do not result in the formation of more or wider apertures (Reeder et al., 2016; Dobritsa et al., 2018). (ii) Combining the reduced INP1 levels with the conditions that normally lead to an increase in the number of apertures (forming four apertures instead of three) leads to the formation of four shortened apertures, indicating that INP1 levels can influence the aperture length, but not the number of pre-specified aperture domains (Reeder et al., 2016). (iii) As described earlier, expression of CrINP1 in the Arabidopsis *inp1* mutant rescued the aperture defects but was not sufficient to generate the aperture morphology characteristic of *C. rubella*. Taken together, these results suggest that INP1 assembles at the PM domains whose number and positions have been already specified through some unknown mechanism prior to the INP1 arrival, implying that there must be other factors acting upstream of INP1 in the process of aperture formation.

14 Mechanisms of Aperture Formation: Factors, Players, and Conjectures

The molecular mechanisms that determine where pollen apertures will be placed, how many of them will be generated, and how they will look are essentially unknown. In the following sections, we review the ideas about factors and processes that could potentially be involved in placement and formation of apertures.

Initially, INP1 is distributed diffusely and fairly uniformly within the dividing MMCs and in the early tetrad-stage microspores. The process of INP1 assembly into three punctate lines at the cell periphery is gradual and is not perfectly synchronised either within individual microspores or between the sister microspores (Dobritsa et al., 2018). Interestingly, despite this apparent lack of coordination in the INP1 assembly, in Arabidopsis, as well as in most other eudicot species with triaperturate pattern, the apertures will eventually develop as characteristic pairs (Figure 4a,c); in each microspore in a tetrad, each of its three apertures becomes aligned with an aperture in one of the sister microspores, leading to the formation of six pairs of apertures per tetrad.

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Development of this particular aperture alignment, as well as a more general finding that aperture placement is typically not random between sister microspores within a tetrad (e.g. all microspores in lily or maize have their single apertures developing at the pole distant to the centre of tetrads) suggested that microspore polarity develops coordinately between the progeny of a MMC. These observations have led to ideas that cues for microspore polarity, and the resulting placement of apertures may be connected to male meiosis or meiotic cytokinesis through which tetrads are generated (Wodehouse, 1935; Huynh, 1976; Sheldon and Dickinson, 1986; Blackmore and Crane, 1998; Ressayre et al., 2002, 2003).

14.1 Meiotic Cytokinesis: Arguments in Favour of its Role in Aperture Specification

Two types of male meiotic cytokinesis are typically recognised in angiosperms: (i) successive, in which a callose-based cell plate is formed after each of the two nuclear divisions of meiosis; and (ii) simultaneous, in which no cell plates are formed until the end of both nuclear divisions, followed by the formation of multiple cell plates that simultaneously partition the cytoplasm around the four nuclei (Penet et al., 2005; De Storme and Geelen, 2013). Many monocots have cytokinesis of the successive type – which is often, but not always – associated with centrifugal cell plate formation, when the cell plate expands outward from the center of the cell to fuse with the plasma membrane at the cell periphery. This type of cytokinesis can lead to the development of several types of tetrads (e.g. tetragonal, linear, decussate, or T-shaped) (Furness and Rudall, 1999; De Storme and Geelen, 2013). In contrast, eudicots typically have simultaneous cytokinesis, which leads to simultaneous formation of multiple cleavage planes and is usually associated with inward-oriented (centripetal) furrowing of the parental callose wall (Otegui and Staehelin, 2004). This type of cytokinesis most commonly generates tetrahedral tetrads, produced through the formation of six cleavage walls (Furness and Rudall, 2004; Matamoro-Vidal et al., 2016).

It was noticed a long time ago that in the majority of the eudicot species with three equatorial apertures (the most common pattern in this clade), centres of the apertures lie close to the points of last contact between the sister microspores in tetrahedral tetrads at the end of meiotic cytokinesis (Wodehouse, 1935). Based on these observations, Wodehouse hypothesised that the geometrical arrangement of microspores in a tetrad (e.g. tetrahedral), which dictates the number of contact points, might correspondingly determine the number of developing apertures and cause apertures on one microspore to be aligned with apertures in its sisters (Wodehouse, 1935). The Wodehouse model suggested that the type of cytokinesis and the direction of the cell plate formation might create positional landmarks for aperture sites.

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This idea was further developed by Blackmore and Crane (1998) and Ressayre et al. (2002), whose models also placed cytokinesis at the centre of aperture ontogeny. The model by Ressayre et al. suggested that aperture patterns in species with a small number of apertures (one to six) are determined by the combination of several factors acting in microsporogenesis: (i) the type of cytokinesis (successive vs. simultaneous); (ii) the number and positions of cleavage walls forming during the cytokinesis, whose placement will define the shape of the resulting tetrad (e.g. tetrahedral, tetragonal, rhomboidal, etc.); (iii) the direction of the cleavage wall formation (centripetal or centrifugal); and (iv) the propensity of a species to form apertures at polar or non-polar positions in microspores. The first three of these factors determine the positions of the last-contact points between the sister microspores or the positions of the sites where the callose wall of the cleavage planes is deposited last – these sites are termed aperture convergence areas (ACAs). The fourth factor (development of apertures at polar or non-polar positions) determines whether apertures will be formed directly at the ACAs or oriented towards them (Ressayre et al., 2002). Based on the combinations of these factors, the model predicted a large number of possible aperture patterns.

In support of the idea that cytokinesis plays a central role in establishing aperture patterns, in multiple species correlations were found between the type of cytokinesis and the aperture number and positions (Ressayre et al., 2003; Matamoro-Vidal et al., 2012, 2016). In addition, several studies reported that in some species with inaperturate pollen microsporogenesis becomes relaxed compared to aperturate species (Albert et al., 2009; Nunes et al., 2009; Matamoro-Vidal et al., 2012, 2016). In these cases, the inaperturate species were found to exhibit increased variations in the type of cytokinesis and/or the direction of cleavage wall formation. This was interpreted as the possible consequence of the removal of selective pressure to maintain a certain type of microsporogenesis once the trait produced through it (i.e. apertures) is lost (Matamoro-Vidal et al., 2012, 2016).

A potential weakness of such correlative studies is that they do not experimentally manipulate the system to test the proposed role of cytokinesis in aperture formation and, therefore, do not allow direct conclusions about the causative mechanism. Furthermore, apertures become visible only after cytokinesis is complete – thus precluding direct correlations between the type of cytokinesis and the aperture pattern in a given tetrad – and even when apertures are present in tetrad-stage microspores, they are not always easy to visualise. Development of techniques for culturing tetrads and imaging them live, coupled with the creation of fluorescent markers, such as INP1–YFP, for labelling aperture positions, may in the future allow observations of this process from start to finish.

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14.2 Meiotic Cytokinesis: Arguments Against Its Role in Aperture Specification

Discoveries of several Arabidopsis mutants defective in male meiotic cytokinesis (*osd1*, *tam-2*, *MiMe*, and *tes/stud*) (Hülskamp et al., 1997; Spielman et al., 1997; d'Erfurth et al., 2009, 2010) or having abnormal aperture number (*lsq*) (Dobritsa et al., 2011) allowed recently a more direct testing of the notion that cytokinesis plays the predominant role in aperture development (Reeder et al., 2016).

Several results of these experiments were inconsistent with this hypothesis: First, in *lsq* plants, an apparently normal cytokinesis led to a normal tetrahedral tetrad arrangement. However, microspores in these tetrads developed abnormal number of apertures – mostly four per microspore, not all of which were aligned in pairs. Secondly, in the *tes/stud* mutants lacking cytokinesis, apertures still developed (albeit supernumerary and irregularly placed and shaped). Finally and most importantly, in cases when the second meiotic division was abolished (*MiMe*, *osd1*, *tam-2*), leading to formation of dyads that had only one centripetally formed cell plate and apparently just one last-contact point between the sister microspores, haploid microspores were still able to develop three normal apertures, while diploid microspores formed more than three (Reeder et al., 2016). Taken together, these results suggest that, at least in Arabidopsis, apertures can develop in the absence of cytokinesis, and the geometric arrangement of microspores in a tetrad and the related number of contact points between sister microspores at the end of cytokinesis are unlikely to be the defining factors in determining whether apertures will develop and how many of them will be created.

14.3 Microspore Ploidy

Pollen ploidy, rather than cytokinesis, exhibited a very strong positive correlation with aperture number (Reeder et al., 2016). In Arabidopsis, pollen of higher ploidy almost always has an increased number of apertures, independent of the way such pollen is generated (e.g. through the formation of tetrahedral tetrads produced by higher-ploidy plants or through dyads formed by the omission of second division). Conversely, pollen of normal ploidy, even when generated through unconventional mechanisms, predominantly produces three normal apertures (Reeder et al., 2016). Similar correlations between ploidy and apertures were previously recorded in some other plant species (Tammes, 1930; Hecht, 1941; Ockendon, 1971; Schifino and Moraes Fernandes, 1987; Warner and Chinnappa, 1990; Mignot et al., 1994), suggesting that this phenomenon is widespread. The mechanism through which ploidy regulates aperture number is unknown. One possibility is that an increased gene dosage associated with an increase in ploidy may change levels of some aperture factors specifying the number

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of aperture domains. An alternative possibility is that the effect is caused not by the ploidy itself, but rather by the concomitant changes in cell size, which typically accompany changes in ploidy (Altmann et al., 1994; Marshall et al., 2012; De Storme et al., 2013). Changes in microspore size may impact characteristics such as mechanical properties of its surface or concentration of aperture factors. So far, attempts to uncouple pollen size from ploidy in order to distinguish between these possibilities have not been successful (Reeder et al., 2016).

14.4 Meiotic Divisions and Spindle Positioning

Several authors previously proposed that the orientation of meiotic spindle (or positions of the microtubule organising centres) influences positions of apertures (Heslop-Harrison, 1971; Dover, 1972; Sheldon and Dickinson, 1983). For example, in the experiments with centrifugation of lily buds at different stages of microsporogenesis, abnormal aperture positioning (e.g. formation of 'colpal islands') was sometimes observed when cells were centrifuged during cell division (Heslop-Harrison, 1971; Sheldon and Dickinson, 1983). Similarly, in both lily and wheat, when buds at meiotic stages were treated with colchicine, the resulting pollen frequently developed abnormal apertures (Heslop-Harrison, 1971; Dover, 1972; Sheldon and Dickinson, 1986). While these results have been largely interpreted as the effect of spindle loss or displacement, other interpretations are possible. The centrifugation could have potentially affected multiple parameters – such as, for example, gradients formed by aperture factors. Also, in both types of experiments the disrupted cell division led to the formation of pollen grains with higher ploidy and larger size – which, as described earlier, are associated with abnormal aperture placement even when cell division occurs and spindles are present.

The results described earlier – that in Arabidopsis both the haploid microspores derived from a tetrahedral tetrad (whose formation involves two perpendicular spindles and six cleavage planes) and the haploid microspores derived from a dyad (which requires a single spindle and a single cleavage plane) develop three equidistant apertures (Reeder et al., 2016) – also call into question the assumption that spindle plays the defining role in positioning of apertures.

However, the process of meiotic nuclear division does appear to be required for the formation of apertures, as well as of exine patterns. In Arabidopsis, the *osd1 tam-2* double mutant completely lacks nuclear divisions and produces a small number of unusually large pollen grains (d'Erfurth et al., 2010). This pollen typically develops poorly formed exine with severely disrupted patterning and no recognisable apertures (Reeder et al., 2016). Although the absence of normal exine prevented direct conclusions about the impact of this mutant combination on aperture formation, we tested

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whether it affected localisation of the essential aperture factor INP1. We found that, although in this meiosis-deficient double mutant INP1 was still successfully delivered to the microspore surface, it was usually unable to form regular punctate lines, instead assembling into random puncta or extremely large blobs (Dobritsa and Reeder, 2017). This suggests that the absence of meiotic divisions may disrupt formation of the PM aperture domains at which INP1 aggregates. This process appears to be linked to meiosis and not specifically to cytokinesis (also absent in the *osd1 tam-2* mutant), since the *tes/stud* mutant, which has meiotic nuclear divisions but lacks cytokinesis, still develops furrow-like apertures (Hülskamp et al., 1997; Spielman et al., 1997; Reeder et al., 2016), indicative of the preserved ability of INP1 to assemble into lines at the PM domains.

14.5 Colpal Shield of Endoplasmic Reticulum

Several electron-microscopy studies done in the 1960s and 1970s have reported that the presumed future-aperture sites (in organisms where such positions could be reasonably predicted for tetrad sections) had sheets of endoplasmic reticulum (ER) lying parallel to and directly underneath of the PM (Heslop-Harrison, 1963, 1968b; Skvarla and Larson, 1966; Echlin and Godwin, 1968a; Dickinson, 1970; Blackmore and Barnes, 1987). This led to a hypothesis that these ER elements act as a shield at these sites to prevent secretion of factors required for exine formation (e.g. components of primexine) from microspores (Heslop-Harrison, 1963, 1968a). Although the 'colpal shield' has been often cited in the literature as an established aperture-promoting factor (Sheldon and Dickinson, 1983, 1986; Scott, 1994), whether ER is always associated with aperture formation and whether, if it is present, it is really required for aperture development, is still not clear. In our studies in Arabidopsis, we sometimes observed straight lines of ribosomes underneath the prospective aperture sites (Dobritsa et al., 2018), which could potentially be indicative of the presence of membranes of rough ER at these positions. Still, no studies have yet demonstrated definitively that these portions of ER are indeed involved in formation of apertures.

14.6 Callose Wall and Additional Callose Deposits

One other critical component of microsporogenesis that is often considered in connection with the process of aperture formation is the formation of the callose wall. There are several pieces of evidence that suggest that, in addition to its role in exine development, the callose wall plays an essential role in development of apertures.

In Arabidopsis, the existing mutations in the *CALS5* gene form an allelic series, with different mutants exhibiting different degrees of loss of the callose wall around the tetrads (Dong et al., 2005; Nishikawa et al., 2005). In

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the strong mutants, such as *cals5-2*, in which formation of the outer callose wall around MMCs is essentially abolished and the internal walls between the microspores are weak, the apertures fail to form (Dobritsa et al., 2018). Also, INP1–YFP, which normally localises to the future-aperture regions at the PM, exhibits abnormal localisation in the *cals5-2* background. In this mutant, INP1–YFP predominantly accumulates at the internal regions of the PM where the intersporal callose walls do form, while its accumulation at the outer regions, where callose wall does not develop, is significantly reduced (Dobritsa et al., 2018). In addition, the INP1–YFP puncta seem to closely trace the outlines of the abnormal intersporal walls that develop in *cals5-2*. These results strongly suggest that formation of the normal callose wall is a prerequisite for formation of the INP1 lines at specific membrane positions.

Apertures form at the sites of distinct membrane ridges which maintain close contact with the callose wall when the rest of the PM separates from the wall (Echlin and Godwin, 1968a; Heslop-Harrison, 1968b; Dobritsa et al., 2018). These regions lack deposition of primexine and, possibly because of that, do not develop exine. There is some evidence suggesting that INP1 lines may be extracellular, forming at the interface between the PM domains and the overlying regions of the callose wall and perhaps participating in keeping these domains near the wall (Dobritsa et al., 2018). However, whether INP1 can directly interact with callose, callose synthase, or with some callose-binding proteins is not known.

Several studies have previously shown that the positions of apertures appear to correlate with the sites where callose either forms last at the end of meiotic cytokinesis (as in the cases where apertures develop in pairs at the sites of the closing of cleavage planes) or where extra knobs of callose, known as *additional callose deposits*, are formed after cytokinesis (Albert et al., 2010, 2011, 2014; Prieu et al., 2017). Additional callose deposits have been found in a variety of species from different families, and although in many cases they co-localise with the positions of the last-contact points, they do not always coincide with these sites or with such features of microsporogenesis as the type of cytokinesis, direction of cell plate formation, or tetrad shape (Prieu et al., 2017). In many cases, the additional callose deposits are very inconspicuous, making it hard to determine whether they are present. However, in some species (e.g. *Typha latifolia*, *Luzula forsteri*, *Dracaena surculosa*, and *Illicium floridanum*) in which additional deposits of callose assume the distinct shape of apertures found in these species and are not located at the intersporal walls, these deposits stand out prominently against the callose background (Albert et al., 2011; Prieu et al., 2017). These observations led to the suggestion that it is the positions of last callose deposition rather than of the last cytoplasmic contact, as suggested by the Wodehouse model, that determine the pattern of apertures (Albert et al., 2011). In wild-type Arabidopsis, in which apertures develop near the positions of the last-contact

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points, the deposition of additional callose at these sites is not apparent. Intriguingly, we recently noticed that in the meiosis-deficient *osd1 tam-2* mutant, in which the aperture factor INP1 assembled not in lines but in large bulging aggregates, prominent callose knobs also developed at these sites (Dobritsa and Reeder, 2017). Although all these findings suggest a possible link between the deposition of additional callose and formation of aperture domains, establishing the cause–effect relationships between these processes and identifying the molecular players that participate in them will require further investigation.

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Related Articles

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