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Review article

Formation of aperture sites on the pollen surface as a model for development of distinct cellular domains

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ABSTRACT

Pollen grains are covered by the complex extracellular structure, called exine, which in most species is deposited on the pollen surface non-uniformly. Certain surface areas receive fewer exine deposits and develop into regions whose structure and morphology differ significantly from the rest of pollen wall. These regions are known as pollen apertures. Across species, pollen apertures can vary in their numbers, positions, and morphology, generating highly diverse patterns. The process of aperture formation involves establishment of cell polarity, formation of distinct plasma membrane domains, and deposition of extracellular materials at precise positions. Thus, pollen apertures present an excellent model for studying the development of cellular domains and formation of patterns at the single-cell level. Until very recently, the molecular mechanisms underlying the specification and formation of aperture sites were completely unknown. Here, we review recent advances in understanding of the molecular processes involved in pollen aperture formation, focusing on the molecular players identified through genetic approaches in the model plant Arabidopsis. We discuss a potential working model that describes the process of aperture formation, including specification of domains, creation of their defining features, and protection of these regions from exine deposition.

1. Introduction

The ability of cells to form specialized domains of the plasma membrane (PM), along with the domains in the nearby cortex and extracellular regions, is essential for a wide variety of cellular and developmental processes, including cell and tissue morphogenesis, cell recognition, intercellular communication and transport, barrier formation, and defense against pathogens [1–10]. Yet, despite the importance of distinct domains for growth, development, reproduction, and disease resistance, relatively little is known about how these sites are established and what makes them special. In plants in particular, the processes that help to establish cell polarity and create specific PM domains are only beginning to be investigated [11–16]. In the past few years, several systems have been developed as models to study the formation of distinct domains in the PM of plant cells, most notably Casparian strips in the root endodermis [17–20], apical and basal domains in the root epidermis [21–23], the secondary wall in xylem vessel cells [24–26], the tips of pollen tubes [27,28], the branches of trichomes [29–31], and the stomata lineage establishment and pavement cells in leaf epidermis [32–35]. Still, despite their tremendous utility, these systems do not encompass the diversity of plant cells that develop polarity and form distinct domains. Therefore, studying additional models could reveal other mechanisms that contribute to these processes. In this review, we introduce pollen apertures as a new and exciting model for investigating the mechanisms that guide formation of specific PM domains and deposition of extracellular structures at precise sites.

To protect male reproductive cells and facilitate pollination, developing pollen grains surround themselves with a complex cell wall. The outer pollen wall, called exine, commonly consists of two layers, the outer sexine and the inner nexine [36]. In most seed plant species, exine is deposited on the pollen surface non-uniformly, with certain surface areas developing into pollen apertures. Aperture sites receive reduced amounts of exine or lack exine entirely, and their morphology and structure differ significantly from the rest of the wall. An aperture may be formed on sexine or nexine, and is correspondingly referred to as ectoaperture or endoaperture [37,38]. In eudicots, compound apertures, consisting of both ecto- and endoapertures, are very common [38]. Apertures serve as the portals for water transport and pollen tube germination, as well as the architectural details allowing pollen to accommodate changes in volume, caused by alterations in the state of pollen hydration [39–44]. Apertures generate specific patterns on the pollen surface, dependent on aperture position (polar, equatorial, or distributed throughout the surface), number (from no apertures to more

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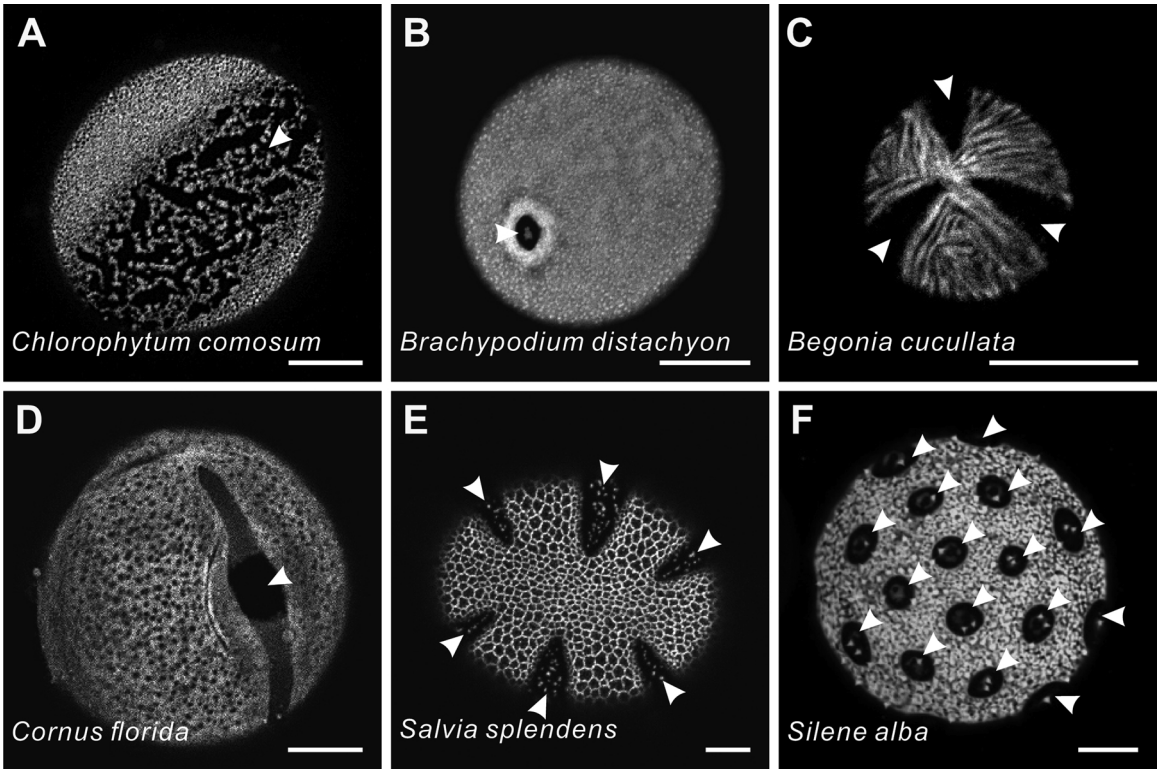

Fig. 1. Diversity of aperture patterns in pollen of different species. Apertures (arrowheads) vary across species in their number, positions, and aspects of morphology (shape, length, width, ornamentation, features of margins, etc.). Scale bars = 10 μm.

than one hundred), and morphology (elongated furrows, pores, or both). Aperture patterns tend to be highly conserved within a species but could differ tremendously between species (Fig. 1), suggesting a tight genetic control over their formation and, potentially, adaptive role of patterns [45].

The presence of apertures on the pollen surface is indicative of the existence of cell polarity in the developing microspores, the precursors of the pollen grains that receive the initial exine deposition. This polarity leads to the establishment of distinct domains in the microspore PM. These membrane domains become specified as aperture domains and exhibit different behavior from the surrounding areas. These domains do not receive deposition of primexine, a microfibrillar matrix that appears around microspores at the early tetrad stage [46]. Primexine is believed to provide a template for the deposition of sporopollenin, the main component of exine, and to guide the development of future exine patterns [47,48]. The aperture domains are also deficient in PM undulation, another early step that appears to be necessary for proper exine formation [46–48]. The absence of these events at the aperture domains eventually leads to these domains receiving less exine deposition than other areas of the pollen surface. How aperture domain sites are established is unknown. The cellular mechanisms that might play a role in aperture formation have been reviewed in previous publications [36,49]. Here we focus on the recent discoveries, via genetic approaches in Arabidopsis, of the molecular factors involved in the formation of aperture domains. We speculate on the possible molecular mechanism of aperture formation and put forward the questions that need to be resolved.

2. Variations in aperture patterns and tools for identifying aperture factors

The aperture patterns on the pollen surface display enormous diversity across species (Fig. 1), and so they are relied upon in the studies of taxonomy and phylogeny of flowering plants. In monocots and basal angiosperms (e.g. Magnoliales, Nymphealeas and Amborella), as well as in gymnosperms (e.g. conifers and Ginkgoales), pollen grains predominantly have a single polar aperture (Fig. 1A-B), usually located at the distal pole (in relation to the center of a developing tetrad of microspores), or develop patterns thought to be derived from the polar aperture: e.g. a single circular furrow running parallel to the equator or passing through the pole; two to four apertures that are distributed within or parallel to the equatorial plane [49–55]. By contrast, eudicots most commonly have three apertures (Fig. 1C-D), equidistantly placed at the equator of the pollen grain, or have patterns derived from the triaperturate pattern (Fig. 1E). In the latter case, apertures can differ in number, but they are still distributed equidistantly and placed perpendicularly to the pollen equatorial plane [49,50]. In addition, pollen of some species displays pantoaperturate pattern, with six or more apertures globally distributed over the pollen surface (Fig. 1F). Although not very common, this pattern occurs in all main groups of angiosperms (e.g. Chloranthales and Austrobaileyales in basal angiosperms, Alismatales, Poales, and Zingiberales in monocots, and Caryophyllales, Ericales, and Rosales in eudicots) [56].

Although aperture patterns have been studied by palynologists, evolutionary biologists, and plant physiologists for a long time, the molecular mechanisms responsible for aperture formation have not been understood. For many developmental processes, isolating mutants defective in these processes and identifying causative genes has been an effective way to explore the underlying molecular mechanisms. Given the variability of apertures in nature, genetic screens in model organisms might be expected to uncover mutants with recognizable differences in aperture patterns, which could allow a powerful insight into the molecular mechanism of aperture formation. Indeed, using this approach, several molecular players involved in this process have been recently discovered in Arabidopsis.

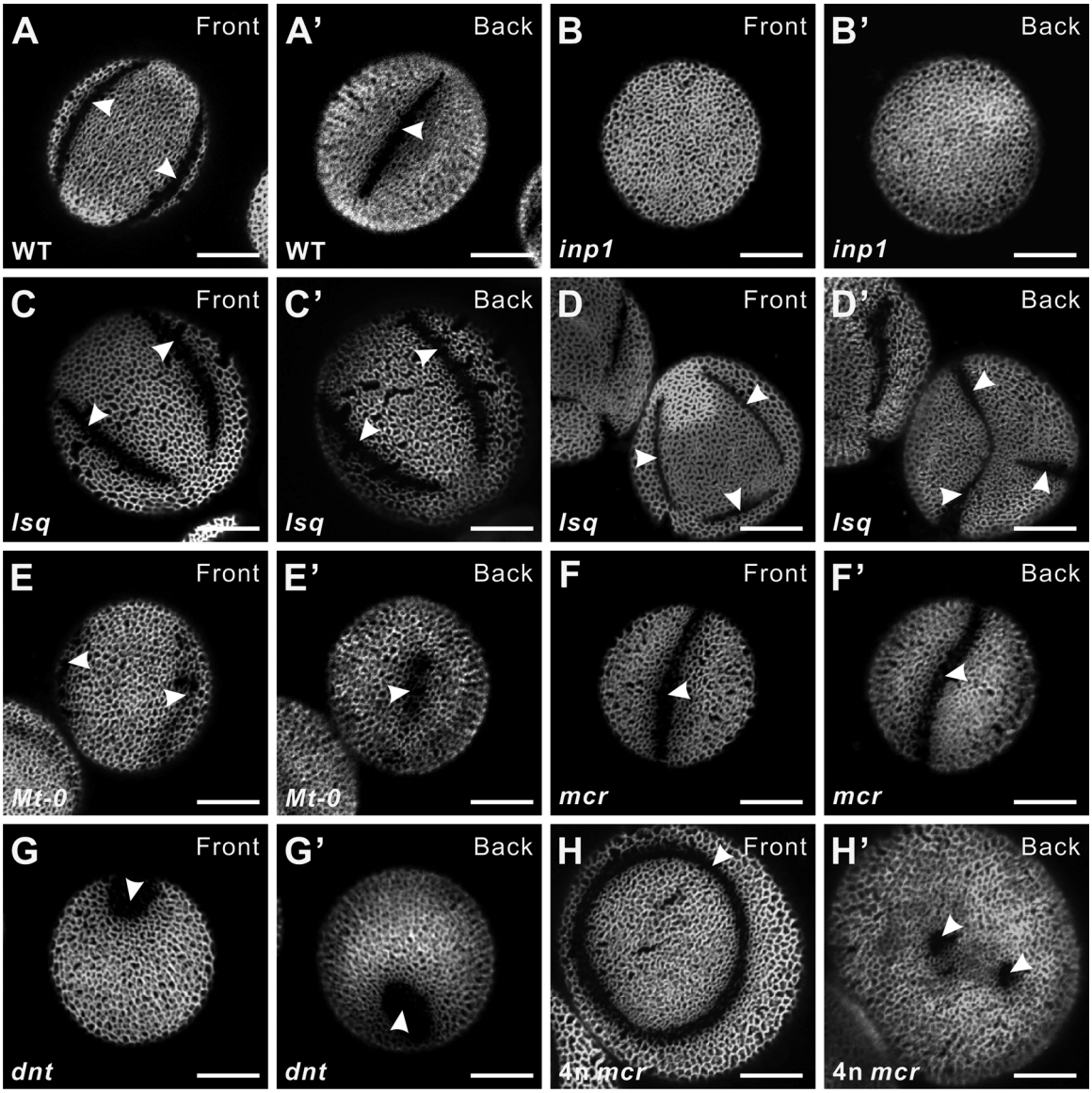

Fig. 2. Pollen aperture patterns of the wild type and aperture mutants in Arabidopsis. (A-A') Wild-type Arabidopsis develops three equidistant apertures on the pollen surface. Front and back views of the same pollen grain stained with auramine O are shown here and in other images as indicated. Apertures here and in other images are indicated with arrowheads. (B-B') *inp1* mutant pollen completely lacks apertures. (C-D') *lsq* pollen (2n) develops more than three apertures, typically forming four (C-C') and sometimes six (D-D') apertures. (E-E') Mt-0 mutant develops unusual apertures that are partially covered with exine. (F-F') *mcr* mutant forms a single ring-like aperture which passes through the poles of the pollen grain. (G-G') *dnt* pollen has two hole-like apertures located at the opposite poles of the pollen grain. (H-H') 4n *mcr* pollen forms a ring-like aperture on one side of the pollen grain and one or two hole-like apertures on the opposite side. Scale bars = 10 μm. Images in (A-A'), (C-C'), and (H-H') are reproduced from [60] and [45] under a Creative Commons Attribution License (CC BY). Images in (D-D') are reproduced with permission from [58] (www.plantphysiol.org); Copyright American Society of Plant Biologists.

2.1. Forward genetic screens discover Arabidopsis mutants with abnormal aperture patterns

Similar to pollen of many other eudicots, pollen of wild-type Arabidopsis develops three long, narrow compound apertures, placed like equidistant meridians around the pollen equator (Fig. 2A-A') [57,58]. Dehydrated triaperturate Arabidopsis pollen always displays an oval shape during dispersal. However, when aperture formation is disrupted, the shape can change into round, rectangular, or triangular. Such changes in pollen shape could be detected at low magnification, with the help of a simple dissecting microscope and without any special treatment of the pollen grains. Thus, pollen shape can serve as a decisive characteristic when screening for aperture mutants [58,59]. Aperture patterns, including aperture position, number, and morphology, could then be determined by examining the entire pollen surface with confocal microscopy [46,58]. Using this strategy, our lab recently conducted several forward genetic screens to isolate aperture mutants in Arabidopsis [58–60].

Two classes of aperture mutants, *inaperturate pollen* (*inp*) and *large and square pollen* (*lsq*), had been identified in a large screen of Arabidopsis SALK T-DNA lines [58]. The *inp* mutants were discovered due to their round pollen shape. Although exine in the pollen of these plants assembles into the normal reticulate pattern, the pollen surface completely lacks apertures (Fig. 2B-B'), resulting in the inaperturate phenotype [58]. Further investigation indicated that the abnormal aperture phenotype of these mutants was caused by mutations in a single gene, *INP1*, the first discovered aperture-specific molecular player (discussed below) [61]. The *inp1* mutants generally exhibit normal fertility, suggesting that in Arabidopsis apertures are not necessary for pollen tube germination, and supporting the view that, even

in the wild type, Arabidopsis pollen tubes can emerge directly through exine, taking the shortest route into the stigma [62]. Inaperturate pollen, although much less common than pollen with apertures, occurs in all major groups of seed plants. For example, pollen grains from some members of Cupressaceae and Taxaceae in gymnosperms, of Orchidaceae and Zingiberaceae in monocots, and of Solanaceae and Ranunculaceae in eudicots are inaperturate, but, like the Arabidopsis *inp1* mutant, develop regular exine [63–68]. This invites the question: could the inaperturate phenotype in any of these species be due to mutations in homologs of *INP1*, or could there be other players whose defects lead to the loss of apertures? In addition, it would also be interesting to know if *INP1* plays a role in development of exineless pollen (such as those of some Zingiberaceae, Musaceae, Cannaceae and Callatrichiaceae), which are sometimes also considered inaperturate or, alternatively, omniaperturate [69,70].

Compared to both wild-type Arabidopsis and the *inp1* mutant, *large and square pollen* (*lsq*) plants produce noticeably larger pollen grains which, when dehydrated, often exhibit rectangular shape. This change in shape is due to the pollen developing more than three apertures, with the predominant phenotype of four equatorial apertures (Fig. 2C-C') [45,58]. The *lsq* mutants were found to be tetraploid and produce diploid pollen. The follow-up study confirmed that the mechanism which specifies the number of aperture domains is sensitive to pollen ploidy [45]. The aperture number exhibits strong correlation with ploidy: in Arabidopsis, the haploid or nearly haploid pollen (1n to ~ 1.5n) consistently develops three normal apertures; the diploid pollen (2n) usually has four or six apertures – with four apertures evenly distributed at the equator (Fig. 2C-C') and six apertures placed on the edges of a tetrahedron (Fig. 2D-D'); and pollen of higher ploidy (≥ 3n) typically develops an even greater number of apertures that often coalesce into ring-like structures [45,60]. This sensitivity of apertures to ploidy extends to other plant families, suggesting that a common mechanism is likely involved [71–74]. However, exactly how the changes in ploidy lead to the changes in the number of specified aperture domains is unknown. Although differences in the expression levels of aperture factors and in the cell size of microspores have been proposed as potential mechanisms [45], so far these possibilities have been hard to test. At the same time, at least in Arabidopsis, the mechanism of aperture number specification appears to be fairly independent of the way the higher-ploidy pollen is generated (e.g. through tetrads, dyads, etc.) [45], suggesting that such factors as the direction of cytokinesis and the number of last-contact points between the sister microspores in a tetrad, previously proposed as important for aperture placement [50,75], probably do not play a major role in this process.

Taking advantage of the existing natural variation in Arabidopsis [76], a forward genetic screen was performed on more than three hundred Arabidopsis accessions [59]. The Libyan accession Martuba (Mt-0) was found to produce pollen that is significantly rounder than normal. This is due to the formation of abnormal aperture sites, which become partially, or sometimes entirely, covered with exine (Fig. 2E-E') [59]. The Mt-0 phenotype, however, is weaker than the completely inaperturate phenotype of the *inp1* mutants. This aperture defect is caused by a recessive mutation in a single gene, *D6PKL3*, encoding a predicted protein kinase, D6 PROTEIN KINASE-LIKE3 (discussed below) [59]. As aperture patterns might influence pollen grain performance, including dehydration, water absorption, hydration, germination, and longevity [44,77], and aberrant aperture phenotypes are not common among natural Arabidopsis accessions (only one out of the 316 screened accessions had abnormal apertures), it is conceivable that the aperture pattern in Mt-0 might have evolved as an adaptation to the dry and hot environment in Libya.

Two more mutants with abnormal aperture patterns have been identified in the recent forward genetic screen performed on an ethyl methanesulfonate (EMS)-mutagenized Arabidopsis population [60]. The *macaron* (*mcr*) mutant develops a single ring-like aperture, which passes through the poles of pollen (Fig. 2F-F') [60]. Further experiments suggested that this single aperture is likely generated by the fusion of two apertures located on the opposite sides of the equator [60]. Thus, the *mcr* mutation leads to changes in aperture number; however, it does not affect their shape or equatorial placement. In contrast, the *doughnut* (*dnt*) mutation changes all aspects of aperture patterns, affecting aperture number, morphology, and positions. Instead of the three elongated furrows, pollen of the *dnt* mutant develops two round, hole-like apertures, which have internal deposition of exine materials and are relocated from the equator to the poles of the pollen grain (Fig. 2G-G') [60]. The aperture patterns similar to *mcr* and *dnt* phenotypes do occur in nature: for example, some species of *Pedicularis* (Orobanchaceae) (e.g. *P. asplenifolia*, *P. gyroflexa*, *P. pulchella*, and *P. cranolopha*) have *mcr*-like apertures, referred to as bi-syncolpate, zonacolpate or ring-like in palynological literature [64,78], whereas *Colchicum autumnale* (Colchicaceae) and multiple species of Bromeliaceae have aperture patterns with some resemblance to the ones in the *dnt* mutant [64]. In the future, the identification of the genes responsible for the aperture phenotype of the *mcr* and *dnt* mutants is expected to provide important insights into the mechanism that specifies aperture patterns.

2.2. Use of mathematical modeling to study aperture formation

To explore biochemical and geometric parameters that may have an effect on aperture patterning, mathematical modeling based on the Gierer-Meinhardt (GM) system of equations was recently performed [60]. The GM model simulates the interaction of two morphogens, a short-range activator and a long-range inhibitor [60,79,80]. This model was able to recapitulate aperture patterns of the wild-type and of higher-ploidy Arabidopsis pollen both in the one-dimensional (1D) domain representing the pollen equator and in the three-dimensional (3D) domain representing the surface of the pollen grain. The model found that pollen size and certain elements of morphogen kinetics, namely the rates of diffusion and decay of morphogens, may influence the aperture number and position. It also predicted some unique aperture patterns, later observed in Arabidopsis. In particular, it predicted the possibility of the normal-ploidy (1n) pollen developing two apertures: this prediction was later confirmed by the discovery of the *mcr* and *dnt* mutants [60]. Interestingly, the aperture patterns in the *mcr* pollen also respond to the induced changes in pollen ploidy in ways predicted by the GM model [60]. In an especially striking case, the 4n *mcr* pollen predominantly forms a ring-like aperture placed on one side of the pollen (corresponding to the distal surface of dyad-stage microspores that give rise to these pollen grains) and one to two hole-like apertures on the opposite side (Fig. 2H-H'), recreating an unusual pattern produced by the GM model in the case of larger pollen that has reduced diffusion of a morphogen.

3. Molecular players contributing to aperture formation

Aperture patterns develop at the tetrad stage, when four sister microspores, which will develop into four pollen grains, are held together after meiotic cytokinesis by their shared callose wall [61,81,82]. Thus, the aperture pattern is determined during microsporogenesis. Several cellular processes associated with microsporogenesis have been proposed to play a role in aperture formation. These processes include meiotic nuclear division [36,83], orientation of meiotic spindles and distribution of spindle microtubules [82,84,85], male meiotic cytokinesis and its related characteristics (cell plate formation, resulting tetrad types, and positions of last-contact points between sister microspores) [50,75,86–89], formation of callose wall and additional callose deposits [83,89–92], and formation of shields of endoplasmic reticulum [93–95]. However, the molecular mechanism of aperture formation is still largely unknown. Recently, the first two molecular players involved in aperture formation have been described in Arabidopsis [46,59,61].

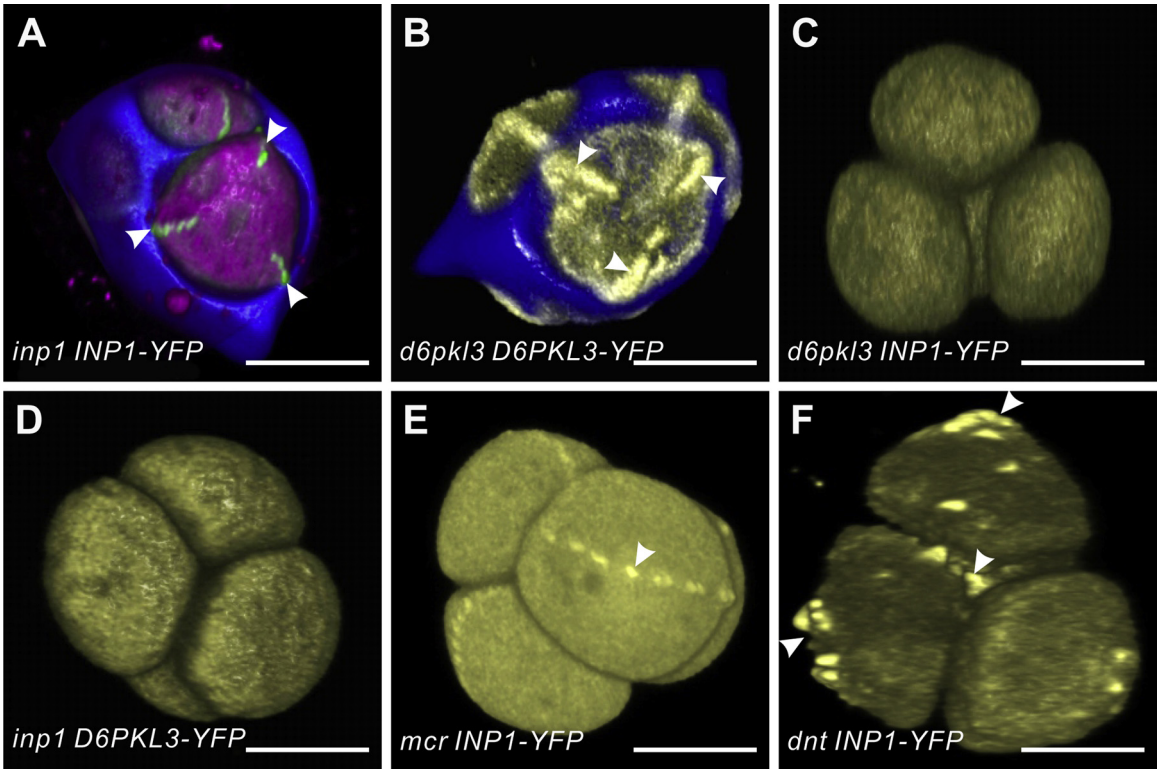

Fig. 3. INP1 and D6PKL3 localize to the aperture domains in tetrad-stage microspores and change their localization in the background of several aperture mutants. (A) INP1-YFP assembles into three equidistant punctate lines (arrowheads) at the periphery of each microspore. (B) D6PKL3-YFP assembles into three equidistant punctate lines (arrowheads) at the periphery of each microspore. (C) In the *d6pkl3* mutant background, INP1-YFP fails to assemble at the aperture domains. (D) In the *inp1* mutant background, D6PKL3-YFP fails to assemble into three lines at the aperture domains. (E) In the *mcr* mutant background, INP1-YFP assembles into a single ring-like punctate line (arrowhead), passing through the microspore poles. (F) In the *dnt* mutant background, INP1-YFP forms two punctate circles (arrowheads) located at the poles of microspore. Scale bars = 10 μm. Images in (A) and (B–D) are reproduced with permission, respectively, from [46] (www.plantphysiol.org) and [59] (www.plantcell.org); Copyright American Society of Plant Biologists. Images in (E–F) are reproduced from [60] under a Creative Commons Attribution License (CC BY).

3.1. *INP1 is an executor of the pollen aperture formation*

INP1 is the first reported aperture gene whose dysfunction leads to the complete loss of apertures. It encodes a novel protein with a single recognizable protein domain, the plant-specific DELAY OF GERMINATION1 (DOG1) domain whose function is unknown [61,96]. INP1 is expressed specifically in the anthers of buds at the microsporogenesis stage [61,97]. Within the male sporogenic lineage, INP1 exhibits dynamic and distinct patterns of subcellular localization [46,61]. Before and during meiotic cytokinesis, INP1 protein is produced by microspore mother cells and is uniformly distributed throughout the cell. At the early tetrad stage, its expression increases, but the protein remains uniformly distributed. However, at the later tetrad stage, INP1 starts gradually assembling into individual puncta at the three equidistant regions of the PM, finally forming three equidistantly placed longitudinal lines at the surface of microspores (Fig. 3A). These lines mark the aperture domains. After the release of microspores from tetrads, INP1 signal soon disappears, suggesting that the protein undergoes rapid endocytosis and degradation [46]. The formation of INP1 punctate lines is not perfectly synchronized either between the sister microspores within a tetrad or even between the three PM domains of one microspore, indicating that each aperture domain attracts INP1 independently [46]. The INP1 lines underlie the sites of future apertures not only in the wild-type microspores, but also in the mutants with abnormal aperture patterns: in these mutants the number and positions of INP1 lines change accordingly – e.g. in the microspores that will develop into the pollen grains with four apertures, INP1 assembles into four lines, whereas in the *dnt* mutant it localizes to two round areas of PM at the poles of the microspore [45,46,60,61]. These results indicate that INP1 always marks positions of future apertures, independently of the locations of these positions.

To understand the role of INP1 in aperture formation, the relationship between the amounts of INP1 and aperture characteristics has been studied. At the low INP1 levels, aperture length, but not the numbers or positions of apertures, is positively correlated with the level of INP1. In the 1n Arabidopsis pollen, apertures become shorter as the level of INP1 gets lower, but the three apertures are still formed [61]. Similarly, in the 2n pollen, which normally forms four apertures, reduction in the INP1 level leads to the formation of four short apertures [45]. In contrast, when INP1 is overexpressed, there are no changes in aperture patterns [45,46], suggesting that, by itself, INP1 is not sufficient to drive formation of new aperture domains. In addition, when the INP1 homolog from *Capsella rubella*, another *Brassicaceae* species, was introduced into the Arabidopsis *inp1* mutant, it restored apertures that had features of the apertures from Arabidopsis, rather than from *Capsella* [98]. Taken together, these results indicate that the sites for the aperture domains of the PM are already established prior to the arrival of INP1. Thus, INP1 appears to act as an essential executor of a predefined aperture pattern, rather than the master regulator responsible for the establishment of this pattern.

Plasmolysis experiments in tetrad-stage microspores suggest that INP1 assembles into lines at the interface between the PM and the overlying callose wall [46]. Presence of the normal callose wall is a necessary prerequisite for the formation of INP1 lines at the correct sites. In turn, the INP1 lines are required for the formation of distinct protruding ridges by the PM at the aperture domains. At the stage when most of the microspore PM separates from the callose wall and forms regular membrane undulations on which primexine, the predecessor of

exine, is deposited, these PM ridges at the aperture domains remain in close contact with the callose wall and become protected from the deposition of primexine. The elucidation of the complex relationship between the PM, callose wall, and INP1 suggests that INP1 may act as a bridge to keep specific PM domains anchored to the callose wall to prevent exine formation at these positions [46]. The proposed role of INP1 in keeping the aperture PM domains near the callose wall is reminiscent of the Casparian strip membrane domain, which also tightly adheres to the overlying cell wall in root endodermis [17]. The Casparian strip domain is specifically marked by complexes of multiple Casparian strip membrane domain proteins (CASPs), suggesting that INP1 at the aperture domains might also be in a complex with other proteins.

Although proteins with similarity to INP1 exist in many angiosperms, INP1 is not well conserved. For example, the corresponding proteins from Arabidopsis and maize share less than 40% sequence identity. It is, therefore, quite remarkable that despite this significant difference between Arabidopsis and maize in the INP1 protein sequence as well as in aperture patterns (in contrast to the three furrow-like apertures of Arabidopsis, maize pollen develops a single aperture in the shape of a round pore [64]), the role of INP1 as an aperture factor is conserved in evolution. This is supported by the finding that, similar to the Arabidopsis *inp1* mutant, the maize mutant defective in the INP1 homolog loses its ability to form an aperture [98]. At the same time, INP1 appears to have limited ability to function in heterologous systems. The interspecies complementation experiments of the Arabidopsis *inp1* mutant demonstrated that only closely related INP1 homologs (from *Capsella rubella* and *Matthiola incana*, members of the *Brassicaceae* family to which Arabidopsis belongs) can correctly localize and appropriately function in Arabidopsis [98]. In contrast, more distant homologs (from tomato, California poppy, and a grass), although likely involved in the aperture formation in their respective species, were unable to restore punctate lines and apertures in Arabidopsis [98]. These data suggest the possibility that in different species the fast-evolving INP1 proteins require species-specific partners which cooperate with INP1 to control aperture formation. Identifying these rapidly evolving INP1 partners will help uncover further details of the molecular mechanism of aperture pattern formation.

3.2. D6PKL3 is a potential creator of aperture domain identity

Another recently discovered molecular player involved in aperture formation is a protein kinase known as D6 PROTEIN KINASE-LIKE3 (D6PKL3) [59]. D6PKL3 belongs to the small D6PK subfamily within the larger AGCVIIIa family of Arabidopsis serine/threonine protein kinases [22,99,100]. The four proteins that comprise the D6PK subfamily have overlapping functions in the control of auxin transport: they all appear to be able to phosphorylate the PIN-FORMED (PIN) auxin transporters and contribute to the development of root and shoot morphology [22,101–103]. However, despite sharing some roles with the other D6PK kinases in the development of vegetative structures, D6PKL3 is the sole member of this subfamily that is involved in the formation of pollen apertures [59]. Notably, auxin transport is closely associated with polarity establishment and PM domain specification in several vegetative systems in plants [21,102], but it remains to be seen whether auxin plays any role in the development of aperture domains.

Like INP1, D6PKL3 localizes to the aperture PM domains in tetrad-stage microspores (Fig. 3B). However, the assembly of D6PKL3 at these sites starts earlier, with the D6PKL3 PM lines already becoming visible during meiotic cytokinesis [59]. This indicates that aperture domains start developing before the full separation of microspores and that D6PKL3 might be involved in an early step of domain formation. Consistent with this idea, the proper localization of INP1 requires the function of D6PKL3 (Fig. 3C) [59]. Additionally, in the transgenic lines which likely overexpress the YFP-tagged D6PKL3, the protein, rather than forming single lines at the three aperture domains, often assembles into two closely positioned parallel lines at each domain. INP1 then also becomes recruited to these parallel lines, leading to the formation of double apertures at each aperture site in mature pollen [59]. These results suggest that D6PKL3 acts upstream of INP1 in regulating aperture formation and could be involved in the initial aperture specification, potentially helping to create the specific features of aperture domains which are used to attract the downstream executors, such as INP1, to those sites.

Unexpectedly, D6PKL3 failed to form peripheral lines in the *inp1* mutant background (Fig. 3D), suggesting that INP1 plays a role either in targeting D6PKL3 to the PM domains or, more likely, in keeping it there. Although D6PKL3 and INP1 depend on each other for correct localization and function, the two proteins do not appear to interact directly, nor could D6PKL3 phosphorylate INP1 [59]. Furthermore, the lines formed by these two proteins at the aperture domains appear to localize to different sides of the PM, with D6PKL3 accumulating at the cytoplasmic side, and INP1 seemingly assembling between the PM and the callose wall [46,59]. Therefore, how these aperture factors cooperate to regulate aperture formation is still unknown.

D6PKL3 may act as a limiting factor in aperture formation, since plants with higher levels of D6PKL3 can form new aperture domains and develop unusual aperture patterns, such as double-line apertures or more than three apertures [59]. Although not a common phenotype, the double-line apertures can be found in nature: e.g. several species of *Passiflora* (e.g. *P. citrine*, *P. costaricensis*, and *P. suberosa*) and *Teucrium* (e.g. *T. chamaedrys* and *T. pyrenaicum*) exhibit them, introducing the possibility that the distinct aperture pattern in these species might be controlled by D6PKL3 homologs or related factors [59,64]. Since the number of apertures is sensitive to pollen ploidy, which, by changing gene dosage, could potentially influence gene expression, the discovery of D6PKL3 invites a hypothesis that the increase of aperture number in the higher-ploidy pollen could be associated with the higher levels of D6PKL3 [45,59]. Still, D6PKL3 does not appear to be the only factor that specifies where apertures would form, since the defective apertures in the *d6pkl3* mutants are still located at the wild-type positions [59].

Both kinase activity and the lysine/arginine-rich (K/R-rich) motif of D6PKL3 are required for proper protein localization of D6PKL3, and its proper function in aperture formation [59]. The fact that D6PKL3 works as an active kinase indicates that phosphorylation has an important role in controlling the aperture pattern, although the aperture factors that serve as the D6PKL3 phosphorylation substrates are still unknown. The K/R-rich motif helps to keep D6PKL3 at the aperture PM domains [59]. In other members of the D6PK subfamily and the larger AGCVIII family, this conserved polybasic motif has been found to interact with anionic phospholipids [59,104]. Consistent with the conserved function of this motif, wild-type D6PKL3 can interact *in vitro* with several phosphoinositides [59]. This interaction is disrupted when lysine and arginine residues in the K/R-rich motif are replaced with alanine. Excitingly, two D6PKL3-interacting phosphoinositides, phosphatidylinositol-4-phosphate (PI(4)P) and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), appear to be specifically enriched at the aperture PM domains [59]. Multiple studies have demonstrated that phosphoinositides can influence establishment of cell polarity through recruitment of membrane-associated proteins to specific PM regions to form distinct domains [15,105,106]. Thus, D6PKL3 might be attracted to or retained at the aperture domains by their interactions with phosphoinositides in the PM. Although INP1 can also bind to PI(4)P and PI(4,5)P2 *in vitro*, if it indeed localizes to the outer leaflet of PM which is devoid of phosphoinositides [107], it would be unlikely to interact with these phospholipids *in vivo*. It might, however, potentially interact with anionic sphingolipids that are present in the outer leaflet and can act as anchors for proteins that target the exterior PM surface [108].

3.3. Potential new aperture factors

Above, we have described two new aperture mutants: *mcr*, with a

ring-like aperture (Fig. 2F-F'), and *dnt*, which forms two round hole-like apertures (Fig. 2G-G'). In the *mcr* background, INP1 assembles into a single circular line (Fig. 3E) which underlies formation of the ring-like aperture. Interestingly, this circular line of INP1 starts developing at the two opposite sites near the equator of each microspore and then fuses at the poles, suggesting that *mcr* has in fact two apertures [60]. The difference from wild type of the INP1 localization in the *mcr* mutant indicates that *MCR* acts genetically upstream of *INP1* and plays a role in specifying the aperture number, but not the long-furrow morphology or the equidistant longitudinal placement of apertures. Similarly, in the *dnt* mutant, INP1 localizes at the two poles (Fig. 3F), consistent with the positions of the hole-like apertures, demonstrating that *DNT* also acts genetically upstream of *INP1*. Unlike MCR, DNT controls multiple aspects of aperture patterns, including morphology, number, and position [60]. Importantly, the abnormal aperture patterns in *mcr* and *dnt* are not caused by the changes in the factors known or hypothesized to affect aperture formation, such as pollen size, ploidy, or direction of male meiosis or cytokinesis during microsporogenesis [60]. Hence, the identification of the genes disrupted in *mcr* and *dnt* will likely result in new clues for understanding the molecular mechanism of pollen aperture formation.

4. Working model for aperture formation

Recent studies have advanced our understanding of the molecular mechanism involved in the formation of aperture patterns on the pollen surface and allowed the creation of the working model of the aperture pathway (Fig. 4). First, MCR and DNT, possibly guided by some unidentified cues, directly or indirectly define certain areas of the PM as the aperture domains, therefore determining aperture patterns of the mature pollen. D6PKL3 is then recruited to the defined aperture PM domains during male meiotic cytokinesis and is kept at these domains by relying on its kinase activity and the K/R-rich motif which helps it interact with phospholipids. The presence of D6PKL3 and phosphoinositides at the aperture domains possibly creates some distinguishing features, making these domains attractive to executor proteins. Finally, at the tetrad stage, the executor INP1, perhaps in a complex with other factors, assembles at the outer surface of the aperture PM domains and creates a bridge which keeps aperture domains tightly tethered to the overlying callose wall. This close contact between the PM and the callose wall prevents or limits exine deposition at these positions, allowing aperture formation.

5. Future perspectives

Even though the identification of the first aperture factors has revealed some details of the molecular mechanism of this process, a lot of questions remain. One of the most interesting questions is how the developing microspores establish the sites for aperture domains and whether these sites are determined coordinately or independently in each microspore in a tetrad. It has been previously proposed that in eudicots with three equatorial apertures and simultaneous male meiotic cytokinesis (Arabidopsis is one of them), the direction of cytokinesis and cell plate formation could determine positions of apertures [50,88,89]. In those species, meiotic cytokinesis leads to the development of three equidistant last points of contact between each microspore and its three sisters, and it was suggested that these sites may serve as the landmarks which determine placement of apertures and the alignment of apertures between sister microspores [50,75,87]. However, the results of recent experiments in Arabidopsis on mutants defective in cytokinesis have called this hypothesis into question [45]; yet no other plausible mechanisms have been proposed in its place.

The phenotypes of the *mcr* and *dnt* mutants place MCR and DNT at the beginning of the aperture pathway, suggesting that these factors are involved in early steps of aperture domain specification. It would be important to identify the corresponding proteins, characterize their functions, and understand what kind of initial cues they may receive in order to place apertures at the correct positions and allow them to develop their proper shape. Also, further investigation into the functions and movements of known aperture factors is needed. Both D6PKL3 and INP1 are produced by the microspore mother cells and initially uniformly distributed, later assembling at the aperture domains. It remains unknown how these proteins are targeted and transported to the aperture domains. INP1, which seems to aggregate at

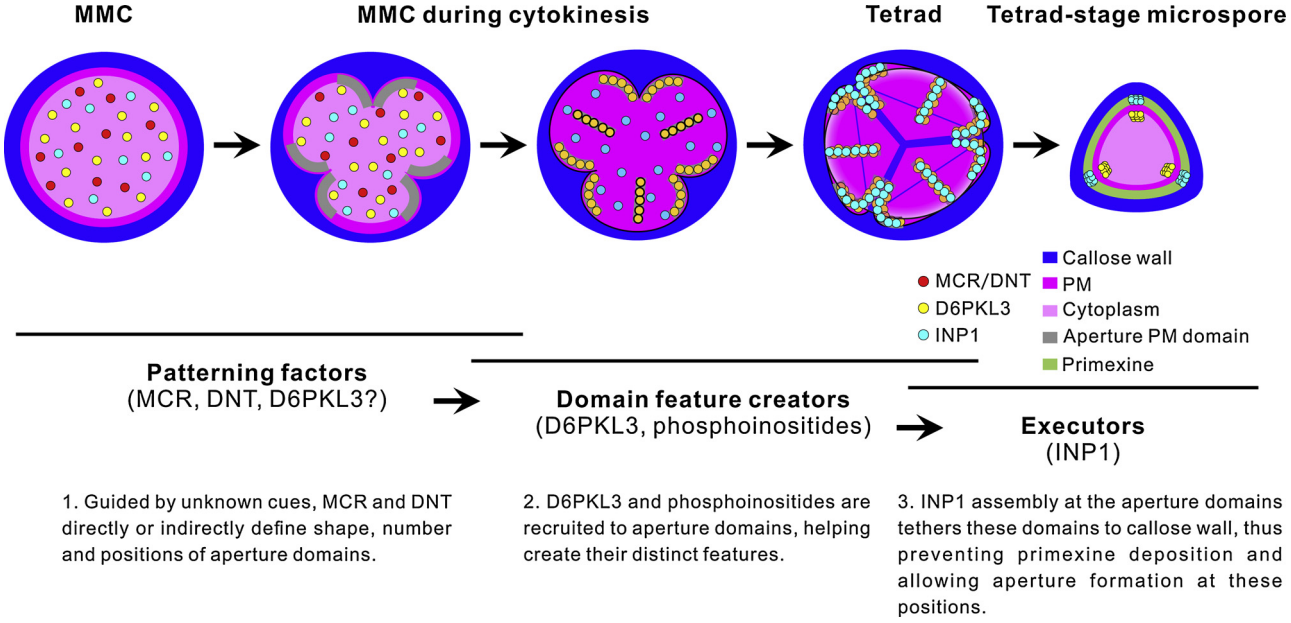

Fig. 4. A working model of the aperture pathway. Callose wall, cytoplasm, PM, aperture PM domains, and primexine are indicated, respectively, in blue, pink, magenta, gray, and green. MCR/DNT, INP1, and D6PKL3 proteins are represented by red, cyan, and yellow circles, respectively. The expression of MCR and DNT at the stages before or during cytokinesis is predicted based on the corresponding mutant phenotypes and the fact that during the cytokinesis D6PKL3 is already visible at the PM domains, suggesting that the aperture domains exist at that time. It is not yet known if MCR and DNT themselves show any distinct localization patterns (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the periplasmic side of the PM, does not have a recognizable signal peptide, so it is unclear what mechanism delivers it outside. The correct localization of INP1 at the aperture domains requires proper deposition of the callose wall on the surface of tetrads [46]. This is reminiscent of several other systems in which plant cell walls appear to constrain the diffusion of proteins located at specialized PM domains. This effect is particularly pronounced for proteins which extend into the periplasmic space and might interact with cell wall or with cell wall-associated proteins [109–111]. The question of whether INP1 interacts directly with the callose wall or whether it relies on other proteins requires further study. In addition, the apparent enrichment of specific phosphoinositides at the aperture domains and their possible interaction with D6PKL3 leads to the questions about the order in which these lipids and proteins accumulate at the PM domains and whether they require each other to remain at these domains. It also suggests a possible role for phosphoinositide-modifying enzymes in creating the particular lipid composition of the aperture domains.

In other plant systems, the processes of PM domain formation have been found to involve, in addition to phospholipids and membrane-associated kinases, several important factors, including receptor-like kinases and their ligands [112–115], Rho-of-plants (ROP) GTPases [2,24–26,116], cytoskeleton and the exocyst [117–120], as well as NADPH oxidases and reactive oxygen species (ROS) [121–124]. Whether any similar factors regulate formation of pollen aperture domains needs to be further investigated.

Finally, the most fascinating and challenging task ahead is to understand how pollen of different species develops species-specific aperture patterns. Identification of aperture factors and elucidation of the aperture pathway in genetically tractable model organisms will likely provide key insights into the steps in aperture formation mechanisms that have been modified in evolution to achieve aperture patterns that vary so tremendously across angiosperms.

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