Formation of pollen apertures in Arabidopsis requires an interplay between male meiosis, development of INP1-decorated plasma membrane domains, and the callose wall

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ABSTRACT
In most plant species, surfaces of pollen grains display characteristic patterns of apertures, formed by the gaps in the pollen wall exine. The aperture patterns are species-specific and tend to be very precise, with pollen of each species usually developing a certain number of apertures placed at distinct positions and acquiring specific morphologies. The precision with which pollen apertures are produced suggests that developing pollen grains possess robust mechanisms that allow them to specify particular membrane domains as the future-aperture sites and to protect these sites from exine deposition. Recently, we demonstrated that formation of apertures in Arabidopsis depends on certain membrane domains attracting a novel protein, INP1, that assembles into punctate lines and helps to anchor these membrane domains to the overlying callose wall. Here we show that in the absence of male meiosis the ability of INP1 to assemble into lines at the pollen surface is compromised. However, INP1 still arrives to the pollen surface and mediates the interactions between the plasma membrane and the callose wall, potentially contributing to the formation of grossly abnormal patterns on pollen surface.

Many cells form distinct domains within their plasma membranes (PM) and extracellular structures. Specialized PM and extracellular domains contribute to a variety of critical processes, including cell morphogenesis, intercellular communication and transport, cell recognition, and barrier formation; however, the mechanisms that select and create such domains are poorly understood. The pollen surface presents a valuable model for studying these questions. In most plant species, pollen grains are covered by the wall exine that is deposited non-uniformly, leaving a certain number of gaps on the pollen surface. These gaps, called apertures, exhibit specific morphologies and develop at specific positions. For instance, in the wild-type Arabidopsis thaliana, each pollen grain forms exactly three furrow-like apertures placed like three equidistant meridians around the pollen equator. Formation of pollen apertures is indicative of the polarity of the pollen surface and of the ability of the pollen PM to develop specific microdomains.

Previously we demonstrated that formation of pollen apertures in Arabidopsis requires the presence of a novel protein, INAPERTURATE POLLEN1 (INP1), which assembles into long punctate lines at the PM domains destined to become apertures (Fig. 1A). This happens during the tetrad stage of pollen development when the four products of male meiosis, the sister microspores, are transiently held together by the surrounding callose wall. After callose wall formation, most of the microspore PM develops characteristic undulations and initiates separation from the callose wall. We recently demonstrated that, at that time, the domains decorated with the INP1 lines form membrane protrusions and remain in the vicinity of the callose wall. These results suggested that aperture formation likely requires a very close interaction between the specific PM domains and the callose wall. We hypothesized that INP1, either directly or indirectly, mediates the close contact between these PM domains and the overlying callose wall and that this proximity is necessary to prevent deposition of primexine, and thus, the future formation of exine at these sites.

It is not understood what determines the number and positions of sites where INP1 will aggregate and what makes these sites attractive to INP1. In the process of aperture formation, INP1 is likely a late-acting factor that recognizes and responds to aperture patterns that have already been established through an unknown mechanism. We have previously demonstrated that the number and positions of domains at which INP1 assembles can be changed by manipulating microspore ploidy or ploidy-related characteristics: in the microspores that have higher ploidy and larger-than-normal size, INP1 assembles into more...
than three lines and, correspondingly, pollen develops more than three apertures. In addition to ploidy, other studies in various species have suggested that such factors as points of last contact between the sister microspores at the end of meiotic cytokinesis (and thus direction of cytokinesis), positioning of meiotic spindles, and the presence of callose deposits added after completion of cytokinesis (‘additional callose deposits’) may play a role in aperture development. However, whether any of these factors are indeed involved in specification of aperture domains and/or formation of apertures is still unclear.

The combination of the tam-2 and osd1 mutations in Arabidopsis causes the disruption of male meiosis and leads to the double mutant producing only a small number of unusually large pollen grains. This double mutant, therefore, provides an opportunity to examine the effects of meiosis on aperture formation and INP1 localization.

Unlike the other higher-ploidy pollen, the tam-2 osd1 pollen typically does not have recognizable apertures and also develops poorly formed exine with severely disrupted, irregular patterns. To test whether the absence of meiosis affects the abilities of INP1 to arrive at the microspore surface and to assemble there into lines, we crossed the previously described INP1pr:INP1-YFP construct into the tam-2 osd1 background and determined the localization of the INP1-YFP fusion protein in developing microspores. We found that, like in the wild type, in the tam-2 osd1 mutant INP1-YFP was successfully delivered to the microspore surface (Fig. 1B–G). Similar to the INP1-decorated sites which formed membrane protrusions in
the wild-type tetrads and the osd1 dyads of microspores,13 the PM sites covered by INP1 in tam-2 osd1 were often anchored to the internal surface of the callose wall (Fig. 1G). However, unlike in the wild-type or in the higher-ploidy microspores that were produced with the help of meiotic divisions,10 in the meiosis-deficient tam-2 osd1 microspores INP1-YFP rarely assembled into obvious lines. Instead, it formed random puncta on the surface of large tam-2 osd1 microspores (Fig. 1B–C), in some cases assembling into extremely large aggregates (Fig. 1C–C). Correspondingly, the plasma membranes in microspores with such puncta and aggregates formed protrusions at irregular positions. These results suggest that the specification of the membrane domains for future apertures and formation of INP1 lines are dependent on the course of normal meiosis. These processes appear to be dependent on meiosis rather than on cytokinesis, as evidenced by the Arabidopsis tetraspetre/stud (tes) mutants, which have the nuclear divisions of male meiosis but lack cytokinesis.20,21 Furrow-like apertures do develop in tes mutants,10 though these apertures are numerous and prone to forming ring-shaped patterns.

In several other plant species, the positions of pollen apertures were found to correlate with the sites where callose was either deposited last at the end of meiotic cytokinesis or where extra callose was formed after the completion of cytokinesis, leading to the development of ‘additional callose deposits’.19–21 Although we have paid attention to the possibility of extra callose being formed at the sites of future apertures in Arabidopsis tetrads, we were unable to detect it in either wild-type tetrads or dyads formed by the osd1 or tam-2 single mutants. Interestingly, in the tam-2 osd1 double mutant microspores, we observed a number of cases in which positions of INP1 aggregates correlated with the areas at the internal surface of callose wall that exhibited increased fluorescence in the same channel as the signal of the Calcofluor White-stained callose wall (Fig. 1D–G). Although such areas were usually recognizable only by a somewhat stronger fluorescence than in the surrounding background (Fig. 1D–F), in several cases the highly conspicuous, unusual structural aggregates at the inside of callose wall were also observed (Fig. 1G). One possible interpretation of these findings is that these areas correspond to additional callose synthesized at the sites where INP1-decorated membrane is closely apposed to the callose wall. This would suggest that some limited (and, therefore, difficult to visualize) deposition of additional callose may indeed take place at the sites overlying the aperture domains in normal Arabidopsis microspores. Our previous results suggested that the presence of normal callose wall is necessary for INP1 assembly at correct membrane positions.13 However, establishing the cause-effect relationships between the INP1 assembly and deposition of additional callose will require further investigation.

**Abbreviations**

INP1 INAPERTURATE POLLEN1
PM plasma membrane

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