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Interactions between pollen tube and pistil control pollen tube identity and sperm-release in the female gametophyte

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Abstract

Flowering plants have immotile sperm that develop within the pollen cytoplasm and are delivered to female gametes by a pollen tube, a highly polarized extension of the pollen cell. In many flowering plant species, including seed crop plants, hundreds of pollen tubes grow toward a limited number of ovules. This system should ensure maximal fertilization of ovules and seed production, however we know very little about how signaling between the critical cells is integrated to orchestrate delivery of two functional sperm to each ovule. Recent studies suggest that the pollen tube changes its gene expression program in response to growth through pistil tissue and that this differentiation process is critical for pollen tube attraction by the female gametophyte and for release of sperm. Interestingly, these two signaling systems, called pollen tube guidance and pollen tube reception are also species preferential. This review focuses on Arabidopsis pollen tube differentiation within the pistil and addresses the idea that pollen tube differentiation defines pollen tube identity and recognition by female cells. We review recent identification of genes that may control pollen tube:female gametophyte recognition and discuss how these may be involved in blocking interspecific hybridization.

Introduction

The pollinated pistil is a system that integrates hundreds of individual cell:cell interactions to achieve maximal seed production. Consider the journey of a single pollen tube: it interacts with several female cell types as it germinates on the stigma, enters the transmitting tissue of the style, turns out onto the ovary surface, grows up a funiculus, enters an ovule micropyle, contacts a synergid cell, and bursts to release its cargo of two sperm(1, 2)(Figure 1, Figure 2A). The two sperm cells then interact with the degenerated synergid cell before fusing with the female gametes (the egg and central cell) to produce an embryo and endosperm within a developing seed (3).

The complexity of the pollen-pistil system becomes apparent when you consider a model like the *Arabidopsis thaliana* (*At*) that contains 50-60 ovules and hundreds of pollen tubes (Figure 1). It is likely that each of the cell:cell interactions listed above (and others) is mediated by multiple pollen tube and female cell proteins and other molecules (e.g.

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carbohydrates). It is also becoming clear that these signaling systems are not discrete or linear, but feedback on each other to optimize reproductive success. For example, it was recently shown that the fusion of sperm cells with female gametes prevents attraction of more pollen tubes to an already fertilized ovule (4-6). Integration of gamete fusion and pollen tube attraction systems ensures that only a single pair of sperm cells is delivered to the female gametophyte and that gamete fusion occurs successfully before an ovule becomes incapable of attracting another pollen tube. These findings suggest the interesting possibility that steps in the flowering plant reproductive process that were thought to be distinct from each other are integrated in ways that significantly enhance reproductive success.

Pollen tube reception is the cell:cell signaling system that results in cessation of pollen tube growth in the female gametophyte, synergid degeneration, and pollen tube rupture/sperm release. Substantial progress has been made in *At* toward identifying female components of this signaling system (reviewed in (7)), however, relatively little is known about how the female gametophyte senses pollen tube arrival or about how the pollen tube prepares for rupture and sperm release. We review recent studies that suggest the pistil induces a specific pollen tube gene expression program that is critical for pollen tube reception. We discuss this as an example of how distinct cell:cell interaction systems are integrated to maximize reproductive success and how differentiation of the pollen tube within the pistil may be essential for species-preferential recognition of the pollen tube by the female gametophyte.

Pollen tubes differentiate in response to growth through the pistil

Pollen tube physiology changes when it grows through floral tissue. For example, pollen tubes must grow through pistil explants to be able to respond to attractants in vitro (8-10). These experiments use a semi-in vitro (SIV) pollen tube guidance assay in which pollen tubes grow through the stigma and a portion of style before growing onto the surface of pollen growth media toward ovules(10). In *Torenia*, where LURE pollen tube attractants were first described(11), the ability to respond to attractants in vitro increases with prolonged growth through pistil tissue(12). However, when LURE binding sites were assessed by incubating pollen tubes with LUREs followed by detection with anti-LURE antibodies, it was observed that, while growth the pistil was required to generate LURE binding sites, prolonged exposure to the pistil did not increase detection of binding sites further (12). These data suggest that the pollen tube senses the pistil environment, that LURE receptor (not yet identified) expression increases as a consequence, and that prolonged exposure to the pistil environment may activate the receptor.

Interestingly, a pair of *At* pollen tube expressed membrane-associated receptor-like cytoplasmic kinases (LIP1 and LIP2) were recently shown to be induced by growth in the pistil and required for the full response to purified LURE proteins in the SIV assay and for pollen tube guidance in the pistil(13). It is not yet clear whether LIP1 and LIP are directly involved in LURE perception, however, these findings underscore the idea that pollen tubes differentiate during growth in the pistil resulting in expression of proteins required to respond to pollen tube guidance cues.

Pollen tube differentiation for sperm release is controlled by three MYBS

Over 1000 genes were detected in the transcriptome of pollen tubes grown in the SIV assay that were not detected in pollen tubes grown in vitro (14). To identify regulators that control pollen tube gene expression in response to the pistil, 26 SIV-induced transcription factors were identified (14). This list included three closely related R2R3-MYB type transcription factors (Subgroup18 (S18): *MYB97*, *MYB101* and *MYB120*) (14); single and double *myb* mutants did not display seed set defects, however triple mutants had a ~70% reduction in seed production (15). *myb* triple mutant pollen grains and tubes develop normally, grow through the pistil, and target ovules nearly as efficiently as wild type. These data suggest that MYB97, MYB101 and MYB120 do not regulate expression of the LURE receptors. However, upon interaction with the female gametophyte, *myb* triple mutants fail to arrest their growth and ~72% of ovules contain coils of pollen tubes within the female gametophyte(15). Synergid cells targeted by these mutant pollen tubes fail to degenerate normally suggesting a loss of communication between pollen tube and synergid cells during pollen tube reception (15). Furthermore, *myb* triple mutant pollen tubes fail to burst and release sperm. These data suggest that MYB97, MYB101 and MYB120 are critical for the pollen tube to exchange signals with the female gametophyte required for successful fertilization.

Pollen tube reception signaling by the female gametophyte

Pollen tube reception requires a number of synergid-expressed genes including *FERONIA* (*FER*) (16), *NORTIA* (*NTA*) (17) and *LORELEI* (*LRE*) (18) (Figure 2A). Loss-of-function mutations in of each of these genes cause the same phenotype: wild type pollen tubes coil in mutant ovules and fail to release sperm. FER is a transmembrane receptor-like kinase of the CrRLK1L family predicted to have pollen tube or female-gametophyte ligand(s) important for pollen tube reception. FER has a malectin-like extracellular domain, so its ligand could be a carbohydrate or glycoprotein (19) (Fig 2A). FER is localized to the filiform apparatus, and is required for NTA, a 7-pass transmembrane Mildew Resistance Locus O family protein, to be re-localized from the secretory system to the filiform apparatus upon pollen tube arrival (17) (Figure 2A-2B). NTA may interact with synergid- or pollen tube-expressed proteins upon relocalization and also contains a calmodulin-binding domain in its cytoplasmic C-terminus, possibly allowing synergid cell perception of calcium oscillations during reception (20) (Fig 2B-2D). *LRE* encodes a glycosylphosphatidylinositol-anchored protein predicted to be associated with the synergid membrane (18) (Figure 2A). The mechanisms by which FER, NTA, and LRE direct pollen tube reception are unknown and it will be important to define how they sense pollen tube arrival to initiate synergid degeneration and pollen tube burst.

Relatively little is known about the pollen tube-expressed genes involved in pollen tube reception and no direct connections between pollen tube and synergid genes have been made. Interestingly, a pair of pollen tube-expressed members of the *FER* family members [CrRLK receptor-like kinases, *ANXUR1* (*ANX1*) and *ANXUR2* (*ANX2*)] may be negative regulators of pollen tube burst. *anx1*, *anx2* double mutants produce pollen tubes that burst very soon after germination (21, 22). Like FER, the ligands for ANX1 and 2 are not yet

known and the nature of ANX contribution to pollen tube reception is unclear because the double mutant phenotype is not informative about their role during synergid interactions.

Auto-inhibited Calcium ATPase9 (ACA9) encodes a pollen-specific calmodulin-binding calcium pump localized to the pollen tube plasma membrane(23). *aca9* pollen tubes have growth defects, however, they can reach ovules in the upper portion of the pistil and ~50% of these enter the ovule micropyle and arrest, but fail to burst (23). This result suggests that ACA9 is important for regulating pollen tube calcium dynamics required for pollen tube burst. Recent live-imaging experiments have shown that calcium concentrations spike in the pollen tube and the synergids as pollen tube burst occurs(20) (Figure 2B-D). It will be very interesting to determine how *aca9* pollen and *nta* (which contains a predicted calmodulin-binding domain) synergid mutants affect these dynamics.

Do MYB target genes interact with the pollen tube reception signaling components?

myb97, *myb101*, *myb120* triple mutant pollen tubes fail to stop growing and burst within the female gametophyte, so the genes regulated by these transcription factors are candidates for pollen tube components of the pollen tube reception mechanism. By comparing the transcriptomes of pistils pollinated with either wild-type or *myb* triple mutant pollen, three main categories of MYB-regulated genes were identified: transporters, small proteins and peptides, and carbohydrate active enzymes (15) (Figure 2A). Many of these genes were found to be in large gene families, potentially explaining why extensive genetic screening in *At* has not identified male pollen tube reception mutants.

Several sugar/proton symporters in the major facilitator family (24) were identified as MYB regulated. *AtSUC7*, *AtSUC8*, and *AtSUC9* are highly induced in wild-type pollen tubes grown in SIV conditions, but are absent in *myb* triple mutants (15). *myb* triple mutants have no pollen tube growth defect in vitro or in the pistil, suggesting these sucrose transporters are not required to import sugars to fuel growth, but may have a specialized function in regulating the pollen tube osmotic state during pollen tube reception.

Multiple small proteins and peptides were found to be potential targets of MYB activation during pollen tube growth (Figure 2A). One of these is a thionin, an 87 amino acid, secreted, cysteine-rich protein (CRP2460, [Silverstein, 2007 #1975]). Thionins have been shown to nucleate membrane pores in artificial lipid bilayers and rat neuronal cells (25); this is an intriguing potential function for pollen tube-expressed thionins given that *myb* triple mutant pollen tubes fail to express these peptides and also have defects in pollen tube burst and initiating synergid degeneration (15). Interestingly, small (130-152 aa) secreted proteins with domains homologous to stigmatic *Papaver rhoeas* self-incompatibility (SI) protein PrsS1(26) were also found to require pollen tube MYBs for expression in the pollen tube (15). In *Papaver* PrsS1 is secreted from the stigma and is bound by PrpS, its pollen-expressed receptor (27). Ligand:receptor interaction blocks germination of self pollen through a mechanism involving calcium as a second messenger and programmed cell death (PCD) (28). The function of *AtS*-protein homologues (SPHs) is unclear (29) and it is surprising to find that they are expressed in pollen tubes in response to growth through the pistil. It will be

interesting to test whether a signaling module similar to that described in *Papaver* is involved in gametophyte interactions that lead to synergid degeneration and pollen tube burst.

The third category of pollen tube MYB-regulated genes encode proteins that interact with carbohydrates, many of which are many are predicted to be extracellular (Figure 2A). These include hydrolases (i.e. Glycoamylase, O-glycosylhydrolase, p-1,3-glucanase, Pectin lyase-like), and pectin methylesterase inhibitors (15). These proteins may be expressed by the pollen tube to modify the cell wall in preparation for pollen tube reception. Alternatively, these proteins may modify the cell wall or other pollen-derived carbohydrates for recognition by the female gametophyte. As mentioned above, FER contains an extracellular lectin domain that could interact with a pollen tube-derived carbohydrate and since *myb* triple mutant pollen tubes behave as if FER signaling is defective, it will be interesting to determine whether pollen tube MYBs regulate production of FER ligands.

MYB-mediated pollen tube differentiation is important for species recognition during pollen tube reception

Pollen tube reception fails in interspecific crosses of *Rhododendron* (30) or *At* (16); pollen tubes of one species overgrow without releasing sperm in ovules of the exotic species. These data suggest that pollen tube reception is an important pre-zygotic barrier to interspecific hybridization. When *At myb* triple mutant pollen tubes enter a wild-type *At* ovule, they overgrow and fail to release sperm (15). These observations lead to the hypothesis that pollen tube MYBs may control expression of the determinants of pollen tube identity during interspecific recognition.

To begin to assess the role of pollen tube MYBs in determining pollen tube recognition, pollen tube reception was investigated in inter-accession and interspecies crosses (Figure 3). The rates of pollen tube overgrowth when *myb* triple and *myb* double mutants were used to pollinate *At* pistils were higher than in any of the inter-accession crosses tested (Figure 3M). *Arabidopsis korshinskyi* (*Ak*) and *Olimarabidopsis pumila* (*Op*) pollen tubes overgrew in *At* ovules more frequently than any of the accessions tested, but the *At myb* triple mutant phenotype was even more pronounced (Figure 3M). These results are consistent with the idea that pollen tube MYBs control pollen tube identity as recognized by the female gametophyte.

When *Ak* or *Op* were used as females in crosses with *At* pollen, increased rates of pollen tube overgrowth were not observed because the percentage of untargeted ovules (likely due to incongruity in pollen tube attraction) was so high that pollen tube reception could not be adequately assessed (Figure 3I,J,M). However, crosses of *At myb* triple mutant pollen onto *Ak* or *Op* pistils showed increases in rates of pollen tube coiling as well as increases in the number of untargeted ovules (Figure 3K-L, M). This finding suggests that MYBs are required for two levels of pollen tube identity factors: 1) core pollen tube identity recognized by exotic female gametophytes and 2) species-preferential identity recognized by the native female gametophyte.

Future work will need to address the mechanisms by which individual MYB target genes confer these identities. One possibility is that MYB-mediate pollen tube differentiation fails when pollen tubes grow through an exotic pistil. Alternatively, MYBs may be properly activated by the exotic pistil and may in turn activate their targets, but the products of these target genes are not recognized by the exotic female gametophyte. In either case, exploration of how the pistil induces MYB97, MYB101, and MYB120 and how the products of their target genes mediate gametophyte interactions provides a path to understand how suitable mates are identified during flowering plant reproduction.

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Figure 1. Pollen tube S18 MYB transcription factors respond to growth through the pistil
 A schematic of an *Arabidopsis thaliana* ovary with 12 ovules (instead of actual 50-60). Pollen grains (red) are germinating pollen tubes on the stigma. Pollen tubes target the female gametophyte (gray). Mature pollen grains (represented in black with two sperm and a nucleus [gray] express MYB101, and lower levels of both MYB97 and MYB120. Transcription of MYB targets is low or absent in pollen grains. Growth through the stigma and style activate transcription of MYB97 and MYB120; the three MYBS activate target genes in the pollen tube. The pollen tube factors that sense the pistil environment and activate MYB expression are not yet known.

Figure 2. S18 MYB regulated gene classes and their putative roles during pollen tube receptions

(A) Schematic of the pollen tube and female gametophyte at the beginning of pollen tube reception. Pollen tube contact with one of the synergid cells is indicated (dotted line). Proteins with roles in pollen tube reception and MYB-regulated gene products are highlighted. Abbreviations: sn - synergid nucleus, vn - vegetative nucleus. (B-C) Schematic shows pollen tube reception between a pollen tube and the synergid that will degenerate. Calcium concentrations are symbolized with a color spectrum (red is high calcium, green is low calcium). (B) FER dependant-NTA relocalization occurs after pollen tube arrival. (C) Synergid degeneration is accompanied by loss of synergid nuclear integrity. (D) Pollen tube burst, Sperm release and double fertilization.

Figure 3.

Pollen tube reception is defective in inter-accession and inter-species crosses. (A-H) *Arabidopsis thaliana* (*At*) *male sterile 1* (*ms1*) pistils from the *Landsberg* accession were pollinated with various pollen donors for 24 hours before imaging pollen tube growth patterns using aniline blue staining and fluorescence microscopy (as in (31)). m - micropyle, arrowheads indicate pollen tubes. (A) *At Col-0* accession, normal entry. (B) *At myb120-3*, coiling. (C) *At myb97-1, 120-3*, coiling and attraction of two pollen tubes. (D) *At myb97-1, 101-4, 120-3*, coiling. (E) *At Cvi-0* accession, two pollen tubes. (F) *At Lc-0* accession, coiling, two pollen tubes. (G) *Ak*, coiling, two pollen tubes. (H) *Op*, coiling, two pollen tubes. (I-L) Emasculated *Ak* pistils were crossed with *At Col-0* (I-J) or *myb* triple mutant (K-L). *At Col-0* tubes show (I) wild-type pollen tube entry, and (J) wild-type entry & one tube failing to enter the micropyle; the majority of ovules were untargeted in interspecific crosses (M). *myb* triple mutants show (K) pollen tube coiling and (L) failure to enter the micropyle (3 tubes) in interspecific crosses. (M) Quantification of pollen tube reception. *Arabidopsis* accessions: *Col-0* (CS1092), *Ler-0* (CS20), *Lc-0* (CS28443), *Cvi-0* (Cs902), *Pn-0* (CS28645), *Ove-0* (CS28590). Related species: *Arabidopsis korshinskyi* (*Ak*) (CS4653) or *Olimarabidopsis pumila* (*Op*) (CS3700). Asterisks denote statistically significant differences from controls by a students t-test, p-value < 0.05.