The Mechanism and Key Molecules Involved in Pollen Tube Guidance

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Abstract

During sexual reproduction of flowering plants, pollen tube guidance by pistil tissue is critical for the delivery of nonmotile sperm cells to female gametes. Multistep controls of pollen tube guidance can be divided into two phases: preovular guidance and ovular guidance. During preovular guidance, various female molecules, including stimulants for pollen germination and pollen tube growth, are provided to support tube growth toward the ovary, where the ovules are located. After entering the ovary, pollen tubes receive directional cues from their respective target ovules, including attractant peptides for precise, species-preferential attraction. Successful pollen tube guidance in the pistil requires not only nutritional and directional controls but also competency controls to make pollen tubes responsive to guidance cues, regulation to terminate growth once a pollen tube arrives at the target, and strategies to stop ovular attraction depending on the fertilization of female gametes.

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INTRODUCTION

Pollen tube guidance is the mechanism whereby the pollen tube (a part of the male gametophyte of flowering plants) is precisely guided to the target embryo sac, an egg-containing female gametophytic tissue (**Figure 1**). Pollen tube guidance is governed by both female sporophytic and gametophytic tissues of the pistil. During the evolution of flowering plants, genes involved in flagella formation (including the flagellar dynein genes) were lost, and the sperm cells of flowering plants could not swim to migrate (3). Instead, the pollen tube cell rapidly conveys a pair of nonmotile sperm cells enclosed by an endocytic membrane of the tube cell. Therefore, precise pollen tube guidance is critical for sperm cell delivery to the target female gametes and for the successful reproduction of flowering plants.

Pollen tube guidance involves multistep controls: Female tissues and cells along the path of the pollen tube successively guide the pollen tube (21, 91). In this review, we separate these multistep controls into two categories: preovular guidance and ovular guidance (**Figure 1**). The preovular guidance navigates all compatible pollen tubes toward the ovary (where ovules reside). Ovular guidance, by contrast, navigates pollen tubes to each target ovule, achieving a precise one-to-one relationship between the pollen tube and ovule. Below, we discuss competency control of

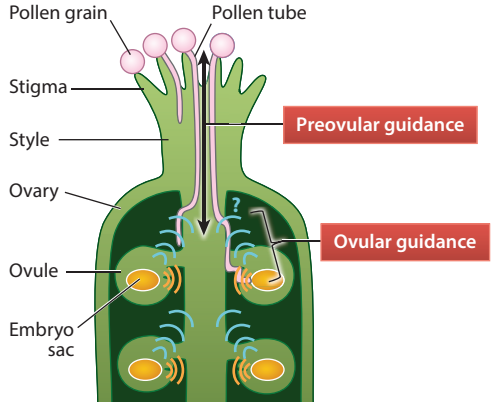

Figure 1

Pollen tube guidance in the pistils of flowering plants. Multistep controls of pollen tube guidance can be divided into two phases, preovular guidance and ovular guidance. Signals from the embryo sac (female gametophyte), shown here in orange, indicate attractant peptides (see **Table 1** and **Figures 2** and **3**, below). Other attraction signals working at a longer range, shown here in blue, have been proposed but remain largely unknown.

pollen tubes for pollen tube guidance, termination of the pollen tube, and cessation of pollen tube attraction as important aspects of pollen tube guidance. We also discuss the mechanism of pollen tube guidance, with a focus on key molecules involved in male-female interactions.

PREOVULAR GUIDANCE AND COMPETENCY CONTROL

Ovules of flowering plants are covered with sporophytic tissues that form a pistil (**Figure 1**). The stigma at the distal end of the pistil is a specialized tissue for receiving pollen grains. After the germination of pollen grains on the stigma, pollen tubes grow straight toward the ovary through the style tissue. Specialized cell files in the style, which form transmitting tracts [e.g., in *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), and *Zea mays* (maize)] or hollow walls [e.g., in *Lilium longiflorum* (lily) and *Torenia fournieri*], define the paths of pollen tubes to approach the ovary. These female cells produce various molecules, as described below. The transmitting tract is indispensable for pollen tube growth and guidance in *Arabidopsis* (17), although genetic ablation of the transmitting tract of tobacco showed that a reduction in the number of transmitting tract cells affects only the initial growth rate (106). Notably, interspecific barriers were diminished in the transmitting-tissue-ablated tobacco (106). Although the structure and size of the pistil differ considerably among species, these sporophytic tissues on the way to the ovule generally play important roles in species recognition and/or self/nonself-recognition, support of rapid growth, directional control, and competency control of the pollen tube (17, 21, 91, 106, 110).

The mechanisms of directional control in sporophytic, preovular guidance remain unclear (40). As attractant molecules, chemocyanin (a small cell wall peptide) and TTS proteins (transmitting-tissue-specific arabinogalactan proteins) have been reported in lily stigmas (63) and tobacco styles (12, 124), respectively. (In this review, polypeptides composed of fewer than 150 amino acids are called peptides, as in Reference 105.) The role of these attractant molecules in preovular pollen tube guidance remains unclear (40). Mechanical guidance in the style has also been observed in lily and *Torenia* species (40). In these species, pollen tubes emerge from the opposite end of the cut style after germinating at the upper (distal) or bottom (proximal) end of the cut style. After germinating in a slit at the middle of the style tissue, pollen tubes grow toward both directions at an equal frequency. A chemotropic directional cue likely does not exist in the styles of these species, and pollen tubes can presumably grow straight along the tissue toward the exit once the entrance into the style has been established.

The concept of competency control of pollen tubes was established when semi–in vitro (also called semi–in vivo) methods were developed in *Torenia* (43) and *Arabidopsis* (90). In these semi–in vitro systems, ovules excised from the ovary are cultivated with pollen tubes growing through a cut, pollinated style. Pollen tube attraction by the ovule and double fertilization occur in cultivated ovules. When pollen grains are germinated on the medium, pollen tube attraction occurs either rarely or not at all. Female sporophytic tissues are thought to enhance the competency of the pollen tube to respond to the attraction signal (43, 90). In other words, pollen tubes require activation or maturation in the pistil tissue for precise pollen tube guidance. This is similar to the capacitation of animal sperms, wherein sperms are activated to acquire fertility on the way to the egg (50).

Okuda et al. (87) examined the relationship between the developmental stage of the pollen tube and the affinity of the pollen tube for an ovular attractant in *T. fournieri*. *T. fournieri* is a unique plant species with a protruding embryo sac that has been used as a model plant species to study pollen tube guidance (41, 43). Ovular pollen tube attractants are LURE peptides (LURE1 and LURE2) of *Torenia* (88) (see Ovular Pollen Tube Attraction, below). LURE peptides belong to a defensin (antimicrobial/antifungal small protein) subfamily of cysteine-rich peptides (CRPs) (105). Immunostaining showed that in vitro pollen tubes that germinated on the medium did not react

to LURE2 peptide even at 12 h after germination, and the LURE peptide consistently did not bind to pollen tubes. At 12 h after pollination, the LURE peptide did bind to the tips of competent semi–in vitro pollen tubes that grew through a 15-mm style (18-mm style at maximum). These results imply that growing through a style of sufficient length confers competency to pollen tubes in *T. fournieri*. Pollen tubes growing through shorter styles could bind LURE2 peptides but were not attracted by it, suggesting that the binding of and response to its peptide are separable steps. Whether the binding of LURE peptide at the tip, as visualized by immunostaining, indicates the binding of LURE peptide to a receptor remains unknown. These physiological changes of the pollen tube are likely indicative of pollen tube maturation.

Pollen tubes change their gene expression profiles during growth through the pistil, as shown by the transcriptomes of semi–in vitro pollen tubes in *Arabidopsis* (96) and *Torenia* (87). For example, various genes involved in intracellular signaling and metabolism were upregulated during pollen tube growth semi–in vitro. Another novel approach is translatome analysis, wherein an epitope-tagged ribosome is expressed with a cell type–specific promoter to isolate mRNAs of the target cell from the whole tissue (75). Through this approach, a novel class of genes enriched in pollen tubes growing in vivo was identified. Importantly, these analyses led to the identification of important genes and mutants of pollen tube function (70, 74, 75, 96). Proteomic approaches in pollen grains and growing pollen tubes are now possible and will allow the detection of more proteins with pollen tube growth regulatory activities and their modifications from small amounts of sample (e.g., 28, 30, 33, 87).

No female molecule that regulates competency acquisition of the pollen tube has been identified. However, many female molecules that stimulate pollen germination and pollen tube growth have been recognized, including ions, water, small compounds such as plant hormones, carbohydrate wall materials, and proteins, including small peptides, as described below. These molecules may be involved in pollen tube maturation.

Upon pollination, dehydrated pollen grains receive various female molecules from the stigma, including water. In flowering plants with a wet stigma, such as lily and tobacco, the surface of the stigma is covered with sugar- or lipid-rich exudates (40), which also contain proteins, including enzymes that degrade polysaccharides and lipids (99). In *Arabidopsis*, which has a dry stigma with long papilla cells, a lipid-rich pollen coat forms a bridge between the pollen grain and a papilla cell during pollination (9). The papilla cell activates secretion and supplies calcium (Ca^{2+})–containing water at the point where the pollen grain is attached (53, 101). Pollen coat molecules induce transcription of a Ca^{2+} pump gene, *autoinhibited Ca^{2+}-ATPase 13* (*ACA13*), in papilla cells and accumulation of ACA13 at the plasma membrane surrounding the pollen tube (53). ACA13 is involved in Ca^{2+} export and is critical for successful fertilization. Boron is also a prerequisite for pollen tube growth (19, 51). In rice, boron is provided from pollen tubes through an efflux transporter, OsBOR4 (113, 114).

Qin et al. (97) recently identified sulfinylated azadecalin (S-azadecalin) in *Arabidopsis* as a molecule that stimulates pollen germination. Characterization of stimulant molecules derived from the pistil tissue of *Arabidopsis* suggested that the molecule is small, hydrophilic, diffusible, and a nonprotein. The authors used pistil extract to purify the molecule and identified it as a molecule of the *m/z* (mass-to-charge ratio) 202.126 ion, which corresponds to the chemical formula of $C_{10}H_{20}NSO$, or S-azadecalin. In addition, chemically synthesized S-azadecalins showed pollen germination activity. S-azadecalins stimulate pollen germination specific to some genera of Brassicaceae but do not affect the pollen tube growth rate.

Brassinosteroids are provided to pollen tubes from female tissues along the path of pollen tubes in *Arabidopsis* (118). Adding 10-μM epibrassinolide to the medium increased pollen germination and the pollen tube growth rate ninefold and fivefold, respectively. Other small molecules include

γ-aminobutyric acid (GABA), which stimulates pollen germination and pollen tube growth via putative Ca^{2+}-permeable membrane channels of the pollen tube (77, 89, 125). GABA is thought to be involved in ovular guidance, but pollen tube attraction activity has not been observed.

Peptides/proteins and glycoproteins are other classes of molecules derived from the female tissue (39). For example, in lily, SCA (stigma/stylar cysteine-rich adhesion) peptide, which belongs to the lipid transfer protein subfamily of CRPs, promotes adhesion of pollen tubes (84, 92). SCA peptide also enhances the chemotropic activity of chemocyanin, possibly by facilitating access of chemocyanin to the pollen tube (63). STIGMA-SPECIFIC PROTEIN 1 (STIG1) of *Solanum lycopersicum* (tomato), which belong to another subclass of CRPs, is abundant in the stigma exudate and stimulates pollen tube growth in vitro (115) and in the pistil (47). STIG1 interacts with a binding partner of the pollen receptor kinase LePRK2 and phosphatidylinositol 3-phosphate; these interactions are also important for the elevation of redox potential by STIG1 (47). A CLV3/ESR-related peptide of *Arabidopsis*, CLE45, stimulates pollen tube growth via two possible receptors in the pollen tube, STERILITY-REGULATING KINASE MEMBER 1 (SKM1) and SKM2 (26). Notably, CLE45 is involved in high-temperature tolerance and stimulates pollen tube growth (26). Various arabinogalactan proteins are present in the extracellular matrix of female tissues along the entire pathway of pollen tubes (e.g., 15, 108). In *Nicotiana*, TTS proteins and 120-kDa glycoprotein (120K), which are arabinogalactan proteins derived from the transmitting tissue, are involved in the stimulation of pollen tube growth, attraction of pollen tubes in vitro, and self-recognition (39). The function of arabinogalactan sugar chains remains unclear, but Lamport et al. (66) have proposed a potential role in storing Ca^{2+} in the extracellular matrix as a Ca^{2+} flux capacitor.

The molecular mechanism of competency control of the pollen tube by female tissues is important to understand pollen tube guidance. Further analyses will require novel methods such as bioassay systems to identify molecules that enhance the competency of the pollen tube or reporter genes that visualize the competency status of each pollen tube. Note that not only factors in the stigma and style but also those provided by ovules can be involved in competency control.

OVULAR POLLEN TUBE ATTRACTION

After entering the ovary, pollen tube guidance is controlled by the ovule (**Figure 1**). An ovary contains approximately 50 ovules in *Arabidopsis*, 500 ovules in *Torenia*, 3,000 ovules in tobacco, and a single ovule in maize and rice. Each ovule is a functional unit of pollen tube guidance that independently navigates directional growth of the pollen tube. The exact range of ovular pollen tube guidance in the pistil has not been clarified. However, in semi–in vitro systems, pollen tubes are guided normally in the cut style and emerge from the cut end of the style without ovaries.

In *Arabidopsis*, pollen tubes tend to emerge from the transmitting tract onto the surface of the septum more frequently at the upper (distal) part of the ovary (49). A similar tendency was observed when a sufficient number of pollen grains was pollinated to the pistil (H. Takeuchi & T. Higashiyama, unpublished data), although it may not necessarily occur when a very limited number of grains is pollinated. Sporophytic mutants defective in ovule and female gametophyte development lost this tendency; pollen tubes still emerged from the transmitting tract but showed little preference for emerging at the upper part of the ovary (49). This suggests that pollen tube guidance by the ovule is effective over the range at which pollen tubes emerge from the transmitting tract of the ovary. Consistent with this idea, semi–in vitro quantitative assays in *T. fournieri* using a microfluidic device were suggestive of long-range attraction activity of the ovule beyond the presumptive attraction range by the synergid cell, as described below (46). Microfluidic devices provide powerful means to study pollen tube growth and guidance under precisely defined

conditions, facilitating studies on pollen tube guidance and its signaling molecules as well as live imaging of plant reproduction (1, 4, 102).

The double-mutant pollen tubes for the endoplasmic reticulum (ER)–localized potassium transporters CHX21 and CHX23 in *A. thaliana* exhibit normal growth in the transmitting tract but defects in emergence from the transmitting tract (79). This is the only reported mutant involved in pollen tube emergence, but how potassium transporters in the pollen tubes regulate competency and/or the ability to respond to attraction cues from the ovule remains unclear. Few studies have examined pollen tube emergence from the transmitting tract, and no female attractant molecules from the sporophytic tissues of the ovule have been identified. Thus, examining pollen tube emergence is important to increase our understanding of long-range pollen tube attraction mechanisms for efficient targeting toward the ovules.

Emerged pollen tubes on the septum surface are attracted to each ovule and enter the ovular micropyle. This process has been intensively studied in the past few decades (21, 110), leading to the identification of pollen tube attractant peptides (**Table 1**). Functional female gametophytes play an essential role in pollen tube attraction to the ovule (98, 104), suggesting that ovular attractants are derived from the female gametophytic cells. The semi–in vitro system of *Torenia* revealed that the synergid cell on the side of the egg cell is the source of pollen tube attractants (43, 45). Similarly, in *A. thaliana*, the synergid cell is thought to play an essential role in pollen tube attraction to the ovular micropyle because a synergid-specific transcription factor, MYB98, is required for synergid cell differentiation and normal pollen tube guidance (59).

Transcriptome analysis of *Torenia* synergid cells (88) and comparative transcriptome analysis using *myb98* mutant ovules (55) indicated that many small secreted CRPs, which are typically 50–100-amino-acid polypeptides (105), are abundantly expressed in synergid cells. Recent studies have shown that CRPs have diverse activities for plant cell-to-cell signaling, including stomatal patterning (68, 109), cell expansion (37), plant fertilization (107), and early embryonic patterning (16). Studies on gene regulatory networks downstream of *MYB98* showed that many genes, including *CRP* genes, from several subfamilies are expressed in synergid cells under the control of MYB98 (93, 94), implying that CRPs may be part of a multistep communication between synergid cells and pollen tubes, including attraction.

In *Torenia*, transcriptome analysis of synergid cells identified two CRPs subgrouped into the defensin-like family as pollen tube attractants responsible for guidance to the ovule (88) (**Figures 2** and **3**, **Table 1**). These attractants, named LURE1 and LURE2, are specifically expressed in the synergid cell and can attract *T. fournieri* competent pollen tubes, which were grown on a semisolid medium through the cut style. Injecting morpholino antisense oligonucleotides specific to LURE1 or LURE2 diminished the frequency of pollen tube attraction by the female gametophyte. TcCRP1, an orthologous peptide of *T. fournieri* LURE1 in the related species *Torenia concolor*, was also identified and showed species-preferential attraction activity (56), which is consistent with a physiological analysis of attraction to the ovule using *Torenia* and *Lindernia* species (42). The amino acid sequences of TfLURE1 and TcCRP1 differ in 8 of 62 residues (**Figure 2**).

To identify pollen tube attractants in *A. thaliana*, genome-wide surveys focusing on defensin-like CRPs were performed with a comparative phylogenetic analysis for orthologous CRPs obtained from one of its closest relatives, *Arabidopsis lyrata* (111). Consequently, pollen tube attractants, which are encoded by a cluster of six paralogous genes, including the *AtLURE1.1–1.5* genes and the pseudogene *AtLURE1.6* (encoding proteins collectively named the AtLURE1 peptides), were identified in *A. thaliana* (**Figures 2** and **3**, **Table 1**). With the exception of AtLURE1.5, which lacks one conserved cysteine, the AtLURE1 peptides attract *Arabidopsis* pollen tubes. The representative AtLURE1.2 peptide attracted *A. thaliana* pollen tubes at a higher frequency compared with *A. lyrata* pollen tubes, indicating that the AtLURE1 peptides

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TfLURE1      GEIP---PEQLRY-----VEFCD-LWSADFS---GSCGDLCKKKWGPNFVGDCDWYASTLWTSGDCVCSEKKKK  62
TcCRP1       GQIP---PEPLRY-----VEFCD-LFSGDFS---GSCDELCKKKRGPNFVGDCDWYASTLWTRGDCVCSEKKKK  62
TfLURE2      SWIPFSKPKRGSYRLESDQERCAYLFPEDEAYAIESCNTRCKRTHGETAFGYCDFTFP-YWTAGECQCWSK---  70

AtLURE1.1    ILIKESSEEERIYPFNPVASPFDPRSLNQILKIG-KIGYCFDCARACMRRDRYIRTCSFERKLCRCSYSHIHHTHG  75
AtLURE1.2    TLINGSSDEERTYSFSPTTSPFDPRSLNQELKIG-RIGYCFDCARACMRRGKYIRTCSFERKLCRCSISDIK----  71
AtLURE1.3    ILINESSDEERTYSFSPTTSPFDPRSLNQELKIG-RIGYCFDCARACMRRGKYIRTCSFERKLCRCSISGIK----  71
AtLURE1.4    ILINESSDEQRIYSFSPTTSPFDPRSLNQELKIG-RIGYCFDCARACMRRGKYIRTCSFERKLCRCSISDIK----  71
AtLURE1.5    VLINGSSDEERTYSFSPRASPFDPRSLNQELKIG-RIGYCFDCARACMRRGKYIRTCSFERKLCRYSISDIK----  71
AlLURE1.4    ILIKESSGEETAYYFNPAASPFDPYSLNQKLKQY-WIGYCFDCARACMRKGKYIKRCNLERRLCRCSISKIH----  71
AlLURE1.5    ILIKESSGKETAYYFNPAVSPFDPYSLNHELKQD-WIGYCFDCARACMRRGKYIKRCNLERRLCRCSISKIH----  71
AlLURE1.6    ILIKESSGEETTYYFNPAASPFDPYALNQELLQQGWVGYCFDCARACMRRKKYIKRCSLERHLCRCSIKDIQ----  72
AlLURE1.7    ILIKESSGEETTYYFNPAASPFDPYALNQELLQQGWIGYCFDCARACMRRKKYIKRCSLERHLCRCSIKDIQ----  72
AlLURE1.9    ILIKESSGEETASYFNPAESPFDPYSLNHELKQD-WIGYCFDCARACMRRGKYIKRCNLERRLCRCSISKIH----  71
AlLURE1.10   ILIKESSGEETAYYFNQAASPFDPYSLNQKLKQY-WIGYCFDCARACMRKGKYIKRCNLERRLCRCSISKIH----  71

ZmEA1        ----GMMMKAPGAAGWVICRAVFEANPQLYFTILRTAGAAAAAATFAACSIAS  49
ZmEAL1       SVCPLVTMVAPGVAGQVISRAAFLANPQLYFAVLHKDGGLAAVRMFAR-----  48
ZmEAL2       --PAVAMMKAPGSGGVLVSRAAFLAKKELYFKLLRTGGVAAAVAALA------  54
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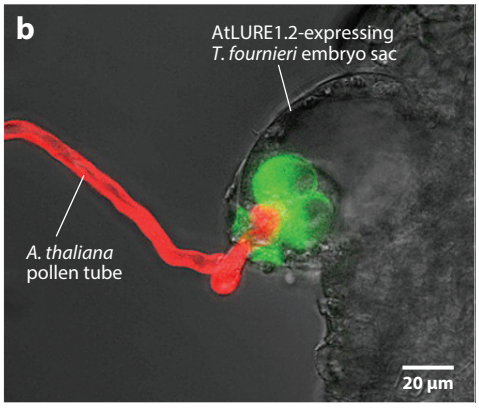

Figure 2

Alignments of pollen tube attractant and related peptides along with an example of overcoming species barriers by heterologous expression of an attractant peptide. (*a*) Alignments of predicted mature LURE peptides of *Torenia fournieri* and *Torenia concolor* (*top*) and *Arabidopsis thaliana* and *Arabidopsis lyrata* (*middle*) and predicted mature EAL family peptides of *Zea mays* (*bottom*). Orange marks indicate cysteine residues, which are conserved in LURE peptides; purple marks indicate other conserved amino acids. The number of amino acids of the predicted mature peptides is indicated at the right. Species specificity or preferentiality in the attraction activity has been suggested for these peptides, likely resulting from differences in amino acid sequences. For additional details on the peptides, see **Table 1**. (*b*) Photograph showing a pollen tube of *A. thaliana* (*red*; *pLAT52::TagRFP*), which was attracted to enter into a protruding embryo sac of *T. fournieri* expressing AtLURE1.2 in two synergid cells (labeled by cytosolic GFP). Note that a single *AtLURE1* gene is sufficient for precise attraction and entrance of the pollen tube into the embryo sac of distantly related species (111). Reproduced from Reference 112 with permission.

are species-preferential pollen tube attractants between two close relatives. AlLURE1 peptide also attracts *A. lyrata* pollen tubes. The amino acid sequences of AtLURE1 and AlLURE1 differ in, for example, 20 of 71 residues for AtLURE1.3 and AlLURE1.4 (**Figure 2**).

Two steps of ovular guidance in *Arabidopsis*—funicular and micropylar guidance—are thought to be separable processes (44). RNA interference knockdown of all AtLURE1 peptides resulted

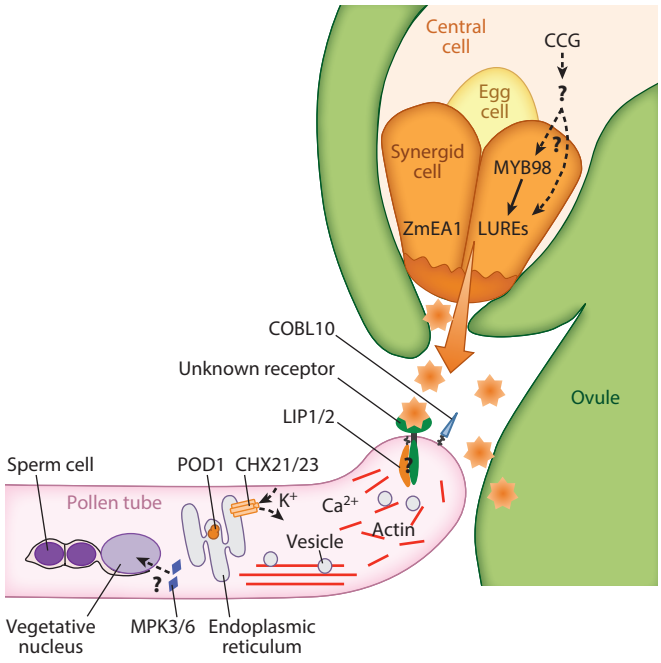

Figure 3

Ovular pollen tube guidance, showing both the female and male molecules involved. LURE and ZmEA1 peptides (*orange stars*) are secreted from two synergid cells on the side of the egg cell.

in reduced pollen tube attraction at the micropyle, consistent with a model in which micropylar guidance is governed by synergid cells. Immunostaining of known micropylar guidance mutants such as *myb98* (59), *magatama* (104), and *central cell guidance* (10) did not detect AtLURE1 peptides at the micropyle. However, immunostaining on the surface of the funiculus (111) and even on the surface of the septum (H. Takeuchi & T. Higashiyama, unpublished data) did detect AtLURE1 peptides beyond the micropyle. AtLURE1 peptides appeared to diffuse the extracellular matrix between cell files of funiculus and septum tissues, along which pollen tubes grow. Given that quantitative analysis of the funicular guidance remains difficult, the range of pollen tube attraction by AtLURE1 and the synergid cell should be examined further.

These LURE peptides from *Torenia* and *Arabidopsis* species are similar defensin-like CRPs but show considerably divergent amino acid sequences (111) (**Figure 2**). Because *Torenia* and *Arabidopsis* are quite distant species among core eudicots, LURE-type CRPs expressed in synergid cells could function as pollen tube attractants in a common but species-specific manner among dicotyledonous plants. In the monocot maize, by contrast, a novel small secreted peptide specific to monocotyledonous plants, *Z. mays* EGG APPARATUS 1 (ZmEA1), is essential for pollen tube attraction to the micropyle (80) and attracts maize pollen tubes, possibly through species-specific interactions with its putative receptor (81, 117). Note that ZmEAL1, one of two other homologs of ZmEA1 in maize, is expressed in the egg cell and is involved in different intercellular communications to prevent antipodal cells from acquiring the cell fate of the central cell (64). By contrast, ZmES4, a defensin family peptide predominantly expressed in synergid cells of maize, induced pollen tube rupture but not attraction, as described below (2).

Ovular guidance cues are also involved in species reproductive barriers. Postpollination but prezygotic interspecific (cross) incompatible mechanisms have been intensively studied to

Table 1 Ovular pollen tube attractant peptides

Peptide name	Species	Peptide types	Amino acids in predicted mature peptides	Expressed cells	Attraction activity	Knockdown	Heterologous expression	Reference(s)
TfLURE1–2	*Torenia fournieri*	CRPs, defensin-like	62–70	Synergid cells	Micropipette and gelatin beads assay	Morpholino antisense oligonucleotide	ND	88
TcCRP1[a]	*Torenia concolor*	CRP, defensin-like	62	Synergid cells	Gelatin beads assay	ND	ND	56
AtLURE1.1–1.6	*Arabidopsis thaliana*	CRPs, defensin-like	71–75	Synergid cells	Gelatin beads assay	RNAi	*T. fournieri* synergid cells	111
AlLURE1.1–1.10	*Arabidopsis lyrata*	CRPs, defensin-like	71–72	ND	Gelatin beads assay for AlLURE1.3	ND	ND	111
ZmEA1	*Zea mays*	EAL family	49	Egg apparatus; predominantly synergid cells	Gelatin beads assay	RNAi and antisense RNA	*A. thaliana* synergid cells	80, 81

Proteins with attractant activity expressed in other sporophytic tissues include chemocyanin (a secreted peptide) (63) and transmitting-tissue-specific proteins (secreted arabinogalactan proteins) (12, 124). Polypeptides composed of fewer than 150 amino acids are called peptides, as in Reference 105. Abbreviations: CRP, cysteine-rich peptide; ND, not demonstrated; RNAi, RNA interference.

[a]Predicted autholog of TfLURE1.

overcome hybridization barriers after cross-pollination between two different species (22). However, the mechanisms are not well understood, in contrast to the molecular mechanisms of intraspecific (self-)incompatibility during pollen germination on the stigma and pollen tube growth in the style. Additionally, although recent studies demonstrated that the extracellular matrix protein of the transmitting tract is involved in interspecific incompatibility in pollen tube growth in tobacco (25, 106), only a few reports have shown interspecific incompatibility in ovular pollen tube attraction (62, 104). Species-specific LURE peptides and ZmEA1 peptides are good candidates for proteins involved in this phenomenon.

In agreement with this hypothesis, in vitro–germinated maize pollen tubes were preferentially guided to the micropylar opening of *A. thaliana* ovules when expressing ZmEA1-GFP in the synergid cell (81). Semi–in vitro *A. thaliana* pollen tubes were also attracted to *T. fournieri* ovules expressing a single *AtLURE1* gene in their synergid cells (111) (**Figure 2**). Unexpectedly, this attraction was very precise and highly reproducible, and the *A. thaliana* pollen tube finally entered the egg apparatus of *T. fournieri* (111), which is similar to pollen tube behavior observed in *T. fournieri* pollen tubes and ovules (40). The identification of this major reproductive barrier molecule directly involved in male-female interactions has increased our understanding of the interspecific incompatibility mechanism and of hybrid breeding by overcoming major barriers.

MALE FACTORS FOR OVULAR ATTRACTION

Several male factors involved in ovular pollen tube attraction have been reported in *A. thaliana* (**Figure 3**). Reviews are available on the details of tip growth of the pollen tube, including the secretion of wall components, cytoskeleton and vesicle traffic control, ion dynamics, and molecular switches such as the Rho of plants (ROP) signaling cascade (13, 31). The first reported male factor essential for pollen tube guidance rather than pollen tube growth involved a pair of potassium transporters, CHX21 and CHX23, as described above (79). In vivo and semi–in vitro experiments indicated that these transporters regulate pollen tube responsiveness to ovular attractants but have little or no impact on growth. This suggests that cellular K^+ homeostasis is required for pollen tube reorientation, possibly by modulating cytosolic cation dynamics (such as Ca^{2+} related to ovular attraction and growth stimulation of the pollen tube) (54, 125) and pH.

POLLEN DEFECTIVE IN GUIDANCE 1 (*POD1*) is also required for normal ovular attraction but not pollen tube growth (72). POD1 is an ER-localized protein that interacts with the ER chaperone CALRETICULIN 3 (CRT3), suggesting that protein folding via POD1 function in the ER is important for pollen tube competency and may control the quality of membrane proteins, including receptors for ovular attractants. These two factors are ER-localized proteins and appear to regulate pollen tube competency for ovular attractants.

A glycosylphosphatidylinositol (GPI)–anchored protein, COBRA-LIKE 10 (COBL10), is localized at the tip of the pollen tube membrane and plays an important role in pollen tube growth and guidance (73). COBL10 requires a C-terminal hydrophobic GPI-anchor signal sequence for its localization at the tip, although the majority of the protein is observed at punctate vesicles. The tip localization appears to be essential for normal deposition of cell wall materials, such as the apical pectin cap and cellulose microfibrils. Consistent with the requirement of the tip localization by the GPI-anchor signal, disruption of the genes *SETH1*, *SETH2* (65), and *ABNORMAL POLLEN TUBE GUIDANCE 1* (*APTG1*) (18), which encode proteins involved in the GPI biosynthetic pathway, affected the tip localization of COBL10 (18, 73). Whereas *seth1* and *seth2* mutants exhibited defects in pollen germination and growth (65), the *aptg1* mutant pollen tube appeared to be nearly normal but showed abnormal guidance around the micropylar opening (18). Besides the male defects, these GPI biosynthesis mutants have general defects such as defective embryo development (18).

Two membrane-anchored receptor-like cytoplasmic kinases, LOST IN POLLEN TUBE GUIDANCE 1 (LIP1) and LIP2, are predominantly localized at the tip of the pollen tube beneath the membrane via N-terminal palmitoylation, and double mutants of *LIP1* and *LIP2* showed defects in pollen tube morphology and guidance in vivo (78). Pollen tube attraction to the AtLURE1.2 peptide was also assessed using semi–in vitro assays and shown to decrease the rate of attracted pollen tubes, but more than 70% of the pollen tubes examined were still attracted, suggesting the existence of one or more redundant factors. Although LIPs cannot directly receive ovular attractants, including AtLURE1 peptides, owing to the lack of an extracellular region, they may form complexes with direct binding receptors of the LURE peptides and play important roles in signal transduction into the pollen tube cytoplasm. Further identification of key factors, including receptors for ovular attractants, is needed to increase our understanding of the precise ovular pollen tube attraction mechanisms involving the ER-localized proteins, GPI-anchored proteins, and receptor-like cytoplasmic kinases.

Pollen tube growth is strongly associated with the dynamics of actin organization and tip-focused Ca^{2+} concentration, and many factors involved in these types of regulation have been identified (31). However, our understanding of actin dynamics and the tip-focused Ca^{2+} in ovular pollen tube attraction (reorientation) remains limited. Two factors, *A. thaliana* MICROTUBULE-ASSOCIATED PROTEIN 18 (MAP18) and MICROTUBULE-DESTABILIZING PROTEIN 25 (MDP25), are proteins with actin filament–severing activity (95, 127). Although the growth of pollen tubes from the *map18* and *mdp25* mutants did not decrease, abnormal targeting to the ovule was observed in vivo when crossed with mutant pollen. These findings demonstrate that proper actin dynamics at the pollen tube tip are essential for pollen tube reorientation and pollen tube growth. The relationship between ovular attractants and reorganization of actin filaments during directional changes in pollen tube tip growth should be explored in the future.

The functions of two mitogen-activated protein kinases, MPK3 and MPK6, in pollen are thought to be involved in ovular guidance, especially funicular guidance, based on a fluorescent-tagged hemizygous complementation strategy that overcomes the embryo lethality of double-homozygous mutants (32). This result is suggestive of competency control for ovular attraction through the MPK3/MPK6 signaling pathway.

TERMINATION OF THE POLLEN TUBE

After a pollen tube arrives at the synergid cell that attracts it, the termination of that pollen tube—that is, the rupture of the pollen tube tip—is critical for sperm cell delivery. The pollen tube appears to grow along or between two halves of the filiform apparatus of two synergid cells rather than penetrating the thickened cell wall of the filiform apparatus (38, 69). The synergid cell is thought to be the point of the concentration maximum of pollen tube attractants. However, if termination is defective, the pollen tube does not stall at the synergid cell and overgrows into the female gametophyte (61, 71, 122).

Genes involved in pollen tube termination have been recently identified (61, 71, 122). The FERONIA (FER) receptor-like kinase, which belongs to the CrRLK1L-1 subfamily, is a key factor for the termination of the pollen tube growth by synergid cells, termed pollen tube reception (27, 48, 100). In *A. thaliana*, the *FER* gene is expressed throughout the plant (except in mature pollen) and is predominantly expressed in synergid cells within the ovules (27). *fer* mutant synergid cells exhibit a pollen tube overgrowth phenotype after pollen tube arrival. Disruption of a pair of the most closely related homologs, ANXUR 1 (ANX1) and ANX2, which are specifically expressed in pollen instead of FER, caused pollen tube rupture before arrival at the synergid cell (7, 83).

CrRLK1L-1 receptors, including FER and ANX, are thought to recognize polysaccharides to monitor cell wall integrity (14, 76). By contrast, the rapid alkalinization factor (RALF) peptide, which belongs to the CRP family, is a ligand of FER and induces a signaling cascade via FER in *A. thaliana* seedlings (37). In addition, FER is required for mechanical signal transduction in seedlings (103). It remains unclear whether FER receives some RALF-like peptides (consisting of more than 30 members in *A. thaliana*) during pollen tube reception and whether ligands of FER originated from the pollen tube or the synergid cell itself for normal pollen tube reception.

FER is responsible for the production of reactive oxygen species (ROS) by NADPH oxidase via ROP small GTPase in both root hair and synergid cells (23, 24). ROS may modulate the cell wall structure and extensibility and activate Ca^{2+}-permeable channels. In *A. thaliana* pollen tubes, ROS production by the pollen-specific NADPH oxidases RbohH and RbohJ is essential for sustained pollen tube growth (6, 60, 67). This process is mediated by ANX and maintains a tip-focused Ca^{2+} gradient (6). Overexpression of ANX1 in the pollen tube enhanced exocytosis, leading to the accumulation of cell wall materials to the apex and abnormal pollen tube growth (6). ROS-induced pollen tube rupture may occur through excess Ca^{2+} influx (23).

Similarly, the synergid-derived defensin-like peptide ZmES4 induced rapid pollen tube rupture by opening the potassium channel in maize (2), and the pectin methylesterase inhibitor ZmPMEI1 appeared to destabilize the cell wall, leading to pollen tube rupture (123). These findings suggest that cell wall modulation is controlled by ROS and other secreted proteins for pollen tube reception through the functions of FER in synergid cells and ANX in pollen tubes, although the dynamics of ROS and cell wall conditions upon pollen tube arrival to the synergid call should be explored further.

Semi–in vitro live-cell imaging has recently highlighted the dynamics of cytosolic Ca^{2+} in synergid cells during pollen tube reception (20, 35, 54, 86). Two properties of Ca^{2+} dynamics in synergid cells have been reported: cytosolic Ca^{2+} oscillations in synergid cells after pollen tube contact, and a Ca^{2+} spike coincident with the pollen tube rupture and receptive synergid cell burst (20, 35, 54, 86). Oscillation of cytosolic Ca^{2+} concentration has been also reported in the tip region of pollen tubes (52), although the functional importance of the oscillation in the synergid cell and pollen tube has not been examined. The Ca^{2+} dynamics during pollen tube reception appear to depend on the FER signaling pathway (86). The cytosolic Ca^{2+} change may control pollen tube reception by the synergid cell and induce pollen tube rupture, although cellular and molecular mechanisms related to cytosolic Ca^{2+} remain unclear. The pollen-specific MYB transcription factors MYB97, MYB101, and MYB120, the first identified set of pollen factors controlling pollen tube reception, regulate gene expression in the pollen tube, including genes encoding small secreted peptides (70, 74). The position of the vegetative nucleus (tube cell nucleus) at the tube tip is involved in appropriate pollen tube termination (126). These findings have provided many clues toward an understanding of the molecular mechanisms underlying pollen-synergid communication for pollen tube reception.

CESSATION OF POLLEN TUBE ATTRACTION

One characteristic property of pollen tube guidance is that each ovule usually receives only one pollen tube (**Figure 4**). In flowering plants with multiple ovules in an ovary, such as *Arabidopsis*, a precise one-to-one relationship is established between pollen tubes and ovules, which may be important for efficient distribution of pollen tubes among ovules and/or for blocking polyspermy.

Multiple pollen tubes of *Arabidopsis* are associated with an ovule in mutants defective in micropylar pollen tube guidance, including *magatama* (104), *myb98* (59), and *central cell guidance* (10). Multiple pollen tubes are attracted to the female gametophyte of mutants defective in pollen

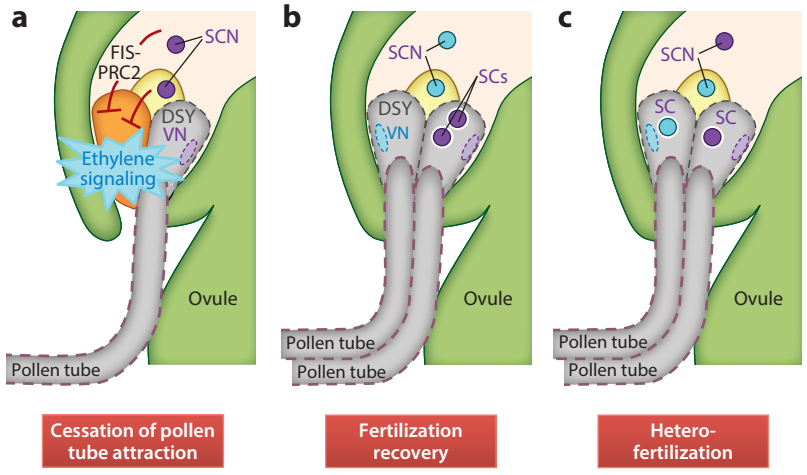

Figure 4

Cessation of pollen tube attraction and fertilization recovery. (*a*) In wild-type plants, gamete fertilization in the egg and central cell independently control cessation of pollen tube attraction. Ethylene signaling in the persistent synergid cell (119) and FIS-PRC2 in the central cell (82) are required for inactivation of the persistent synergid cell and cessation of pollen tube attraction. (*b*) If delivered sperm cells (SCs) are defective in gamete fusion, pollen tube attraction continues (5, 57). Wild-type sperm cell nuclei (SCNs), which are delivered by the second set of pollen tube and synergid cell, then rescue double fertilization (57). This phenomenon is called fertilization recovery (57). (*c*) If the first pollen tube results in a single fertilization of either female gamete, a second pollen tube attraction and reception occur for fertilization recovery of the remnant female gamete. This recovery induces hetero-fertilization (82). Dashed lines indicate the degenerated status of cells and nuclei. For details of female cells in the ovule, see **Figure 3**. Nuclei in female cells are not shown. Additional abbreviations: DSY, degenerated synergid cell; VN, vegetative nucleus.

tube termination (reception), including *feronia/sirene* (48, 100) and *lorelei* (8, 116). A phenotype of more than one pollen tube associated with an ovule is called polytubey (5). The above mutants suggested that polytubey blocking is exerted after the completion of pollen tube termination, although a transient blocking (repellent) signal to prevent the approach of other tubes might be exerted earlier, during ovular guidance of the first tube, as discussed below.

Cessation of pollen tube attraction was recently shown to be controlled by gamete fertilization (5, 57, 82) (**Figure 4**). *GENERATIVE CELL SPECIFIC 1/HAPLESS 2* (*GCS1/HAP2*) genes encode a sperm cell plasma membrane protein required for gametic fusion (85, 120). When sperm cells of *gcs1/hap2* mutants were released into the female gametophyte, they stayed between the egg and central cells but did not fuse with these female gametes (36). Such ovules receiving a *gcs1* pollen tube maintained the attraction activity of the pollen tube (82), consistent with immunostaining showing that AtLURE1 peptides continued to exist in unfertilized ovules receiving a *gcs1* pollen tube but not in fertilized ovules (H. Takeuchi & T. Higashiyama, unpublished data). The second pollen tube was then attracted by the ovule that failed in fertilization with the first pollen tube (5, 57). This phenomenon was also confirmed in two other male mutants, *duo1* and *duo3*, in which sperm cells are defective in fertilization (5, 57). In rare cases, three or four pollen tubes were received by the ovule, but how two synergid cells receive more than two pollen tubes remains unknown (5). The frequency of ovules receiving the second pollen tube reached 77% among ovules accepting a *hap2* mutant pollen tube (57). Only ovules that had failed in fertilization by receiving a *hap2* pollen tube attracted the second pollen tube (5, 57). As discussed above, it appears

that cessation of pollen tube attraction does not occur in an ovule receiving a *gcs1/hap2* pollen tube, although it cannot be excluded that reactivation of the persistent synergid cell occurs in such an ovule. Note that the number of pollen tubes attracted and received by the ovule is strictly controlled by each ovule. The second pollen tube was gradually received from 16 to 28 h after pollination (57), and excess amounts of pollen grains were required for the second pollen tube attraction (58).

Precise in vivo fertility data obtained to examine pollen tube reception in all ovules in an ovary and live-cell imaging in the semi–in vitro system of *Arabidopsis* revealed that the second set of pollen tube and synergid cell could rescue failed fertilization by the first pollen tube (57) (**Figure 4**). The persistent synergid cell rapidly lost nuclear integrity 20 h after pollination (5), but if gametic fusion has not been completed, the persistent synergid cell maintains its function to receive a pollen tube for double fertilization. This unique type of polytubey is also called polysiphonogamy, in the sense that more than one pollen tube is involved in sperm cell delivery and syngamy (57). Observations of two pollen tubes received by a single ovule have been reported in at least 13 other species (57), and three pollen tubes have been reported in *Amborella trichopoda* (121), which has three synergid cells per ovule (29). Fertilization recovery using the persistent synergid cell may generally occur in flowering plants, and the chances for recovery may depend on the number of synergid cells in an ovule.

Cessation of pollen tube attraction is regulated by gametic fertilization, but fertilization in flowering plants is regulated by double fertilization, wherein two sperm cells fertilize two different female gametes. Both fertilizations in the egg and central cell control cessation of pollen tube attraction independently (82). Gamete fusion (plasmogamy) was not sufficient to exert blocking polytubey in the central cell, because the second pollen tube was attracted in an ovule defective in karyogamy of the central cell (82). In two mutants that induce single fertilization, *cdka;1* and *kokopelli* (34), the second pollen tube was still attracted, although the attraction activity was lower in ovules, one of which was fertilized by female gametes (82). Fertilization recovery also occurred, resulting in hetero-fertilization (82) (**Figure 4**), which has been observed for many years; however, how an ovule is fertilized by two pollen tubes remains unclear. Fertilization recovery after single fertilization may be one of the mechanisms for hetero-fertilization; moreover, hetero-fertilization can be induced using a mutant causing single fertilization (82).

Only a few molecules are known to be involved in signaling the cessation of pollen tube attraction (**Figure 4**). One of these, ethylene, is a plant hormone involved in senescence. Fertilization induces ethylene signaling, which is perceived by the persistent synergid cell for programmed cell death (119). EIN3, a transcription factor for ethylene signaling, and EIN2, an ER-localized membrane protein upstream of EIN3 in the synergid cell, are required for synergid cell death to block polytubey (119). Fertilization-independent seed (FIS)–class Polycomb-repressive complex 2 (FIS-PRC2) proteins in the central cell are also critical for blocking polytubey after gamete fusion (82). However, how these signals are integrated for downregulation of the activity of persistent synergid cells and for cessation of ovular pollen tube attraction should be explored in the future. A restart of Ca^{2+} oscillation was also reported in the persistent synergid cell after double fertilization (35). The dynamics of LURE and ZmEA1 peptides after fertilization remain unknown.

The final cessation of pollen tube attraction depends on double fertilization in *Arabidopsis*, but a time delay (possibly up to a few hours) occurs between the beginning of the first pollen tube approach to an ovule and the completion of double fertilization in the ovule. A transient blocking mechanism to inhibit the second pollen tube's approach for several hours may exist, which is also supported by the behavior of pollen tubes in the semi–in vitro system (90) and time course analysis of fertilization recovery (57). Whether a repellent of the pollen tube exists remains unknown. Direct live imaging of pollen tubes in the deep pistil tissue is possible (11, 100). Advances in

these novel methods and technologies to visualize cell behavior and molecules in vivo will provide significant insights into this issue.

SUMMARY POINTS

1. During the preovular guidance step, the pollen tube receives various molecules from female tissues, including ions, small molecules, peptides, and glycoproteins, which stimulate pollen germination and pollen tube growth. Both chemotropic and mechanical guidance are likely involved in directional control. This step also contributes to the control of pollen tube competency to respond to ovular guidance.
2. The synergid-secreted peptides, including LUREs and ZmEA1, are key attractants for ovular pollen tube attraction in a species-specific manner. LURE-type peptides with species-preferential or species-specific activity mediate the final step of pollen tube attraction and species recognition in dicotyledonous plants.
3. Male factors essential for pollen tube attraction but not growth have been identified. The ER-localized proteins and GPI-anchored protein may modulate competency control for response to ovular attractants. The receptor-like cytoplasmic kinases may form a complex for receptors of ovular attractants.
4. The FER receptor-like kinase pathway plays a key role in pollen tube termination (reception) by producing reactive oxygen species and modulating cytosolic Ca^{2+}.
5. Final cessation of ovular pollen tube attraction depends on double fertilization, which involves ethylene signaling in the persistent synergid cell and FIS-PRC2 in the central cell. Failed gamete fertilization after pollen tube arrival is recovered via attraction of the second pollen tube, which also induces hetero-fertilization.

FUTURE ISSUES

1. Key molecules for pollen tube guidance have not been identified, including molecules for competency control, receptors of attractant peptides, ovular sporophytic attractants, male factors for pollen tube termination, and a putative repellent of the pollen tube to establish a one-to-one relationship between pollen tubes and ovules.
2. In vivo imaging will provide important insights into the actual behavior of all pollen tubes and the dynamics of diffusible signaling molecules in the pistil. Such spatiotemporal information is needed to elucidate the mechanism of pollen tube guidance in vivo.
3. Interdisciplinary research will lead to breakthroughs in the study and control of pollen tube guidance. For example, engineering technology provides microfluidic devices for precise in vitro studies, and synthetic chemistry enables structure-activity relationship studies on bioactive molecules.

DISCLOSURE STATEMENT

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