Advanced Review

Pathfinding in angiosperm reproduction: pollen tube guidance by pistils ensures successful double fertilization

Ravishankar Palanivelu* and Tatsuya Tsukamoto

Sexual reproduction in flowering plants is unique in multiple ways. Distinct multicellular gametophytes contain either a pair of immotile, haploid male gametes (sperm cells) or a pair of female gametes (haploid egg cell and homodiploid central cell). After pollination, the pollen tube, a cellular extension of the male gametophyte, transports both male gametes at its growing tip and delivers them to the female gametes to affect double fertilization. The pollen tube travels a long path and sustains its growth over a considerable amount of time in the female reproductive organ (pistil) before it reaches the ovule, which houses the female gametophyte. The pistil facilitates the pollen tube's journey by providing multiple, stage-specific, nutritional, and guidance cues along its path. The pollen tube interacts with seven different pistil cell types prior to completing its journey. Consequently, the pollen tube has a dynamic gene expression program allowing it to continuously reset and be receptive to multiple pistil signals as it migrates through the pistil. Here, we review the studies, including several significant recent advances, that led to a better understanding of the multitude of cues generated by the pistil tissues to assist the pollen tube in delivering the sperm cells to the female gametophyte. We also highlight the outstanding questions, draw attention to opportunities created by recent advances and point to approaches that could be undertaken to unravel the molecular mechanisms underlying pollen tube–pistil interactions.

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INTRODUCTION

In flowering plants, pollen must overcome two challenges before it can deliver the sperm cells for fertilization. First, upon release from the anthers, pollen has to reach the female reproductive organ, the pistil. Pollen accomplishes this task either by dehiscing onto the pistil (typical in bisexual flowers) or with assistance from other agents such as wind or insects (common in unisexual flowers). Second, after reaching the stigma, its landing platform on the pistil, the pollen has to deliver the sperm to the egg. This is a challenging journey, as the egg is typically located far from the stigma and inside the female gametophyte that is enclosed within an ovule. To realize this task, the pollen grain forms a pollen tube through which the sperm cells are transported.[1] Pollen tubes invade the pistil and migrate past stigma, style, and transmitting tract tissues before emerging into the ovary (Figure 1(a)). Subsequently, the pollen tube migrates up the funiculus, enters the ovule, and targets the female gametophyte to affect double fertilization (Ref 2; Figure 1(b) and (c)). Thus, the pollen tube travels a long path (e.g., several centimeters in large flowers[3] and several hundred centimeters in the maize style, the 'silk'), interacts with seven different pistil cell types (stigma, style, transmitting tract, septum, funiculus, integument, and synergid cell), and sustains

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*Correspondence to: rpalaniv@ag.arizona.edu
School of Plant Sciences, University of Arizona, Tucson, AZ, USA

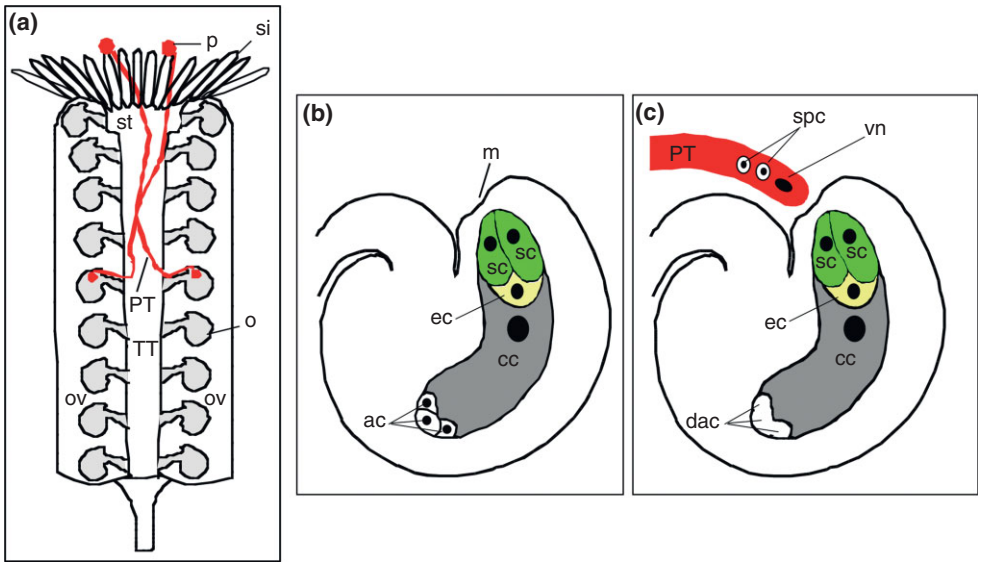

FIGURE 1 | Pollen tube growth and guidance to ovule micropyle. (a) Diagram of pollen tube growth within an *Arabidopsis* pistil. Pollen grains (p) on the stigma (si) germinate and extend pollen tubes (PT, red) through the style (st) and transmitting tract (TT) before entering one of the two ovary (ov) chambers to target an ovule (o). (b) Diagram of a female gametophyte within an ovule. m, micropyle; ac, antipodal cells. The central cell has a homodipoid nucleus, and therefore, its nucleus is larger than the haploid nuclei in the synergid, egg, and antipodal cells. (c) Diagram showing a pollen tube (PT) approaching the ovule containing a mature female gametophyte. Prior to pollen tube arrival in the ovule micropyle, all three antipodal cells degenerate (dac). spc, two sperm cells inside a pollen tube; vn, vegetative nucleus (nucleus of the pollen tube cell); sc, synergid cell; ec, egg cell; cc, central cell.

its growth over a long duration[4] prior to completing its journey.

The multicellular female gametophyte forms from meiosis of the megaspore mother cell (megasporogenesis) followed by development of one of the four meiotic products that survives (megagametogenesis). In the polygonum type of megagametogenesis, which is common in most flowering plants, the lone surviving meiotic product undergoes three mitotic divisions that produce a female gametophyte with eight haploid nuclei. These nuclei migrate to specific positions within the female gametophyte, cellularize, and differentiate into seven cells (Figure 1(b)): three antipodal cells, two synergid cells, an egg cell, and a central cell with two polar nuclei.[5,6] In many plants, including *Arabidopsis thaliana* (henceforth referred as *Arabidopsis*), prior to pollen tube arrival, the two polar nuclei fuse to form the homodiploid central cell and the three antipodal cells degenerate, resulting in a four-celled female gametophyte (Figure 1(c)).

Typically, a single pollen tube enters the ovule through the micropyle, terminates its journey within the female gametophyte in the synergid cell, and bursts to release two sperm cells, one of which fuses with the egg cell to form an embryo, while the other fuses with the central cell to form the progenitor of the endosperm.[7,8] Each fertilized ovule develops into a seed and the pistil with many fertilized ovules matures into a fruit. The pistil facilitates the pollen tube's journey by providing multiple, stage-specific, nutritional, and guidance cues along the pollen tube path. The goal of this review is to summarize our understanding of the cues generated by the pistil tissues to assist the pollen tube in delivering the sperm cells to the female gametophyte. We also highlight the outstanding questions, draw attention to opportunities created by recent advances and point to approaches that could be undertaken to characterize the molecular mechanisms mediating pollen tube–pistil interactions. Where appropriate, we refer the reader to several recent review articles that discuss in greater detail certain aspects of pollen tube growth that are beyond the scope of this review.[9–13]

GETTING READY FOR THE JOURNEY: POLLEN ADHESION TO THE STIGMA

The pistil's stigma captures pollen from anthers, the wind, or insects (Figure 2). Pollen–stigma adhesion is strong, highly selective, and rapidly established, providing stigmas with an opportunity to promote and ensure fertilization only by the appropriate pollen.[14] The pollen cell surface and extracellular matrix (ECM) of the stigma play an important role in mediating pollen adhesion. Pollen adhesion is beyond the scope of this review, and we refer the reader to a recent

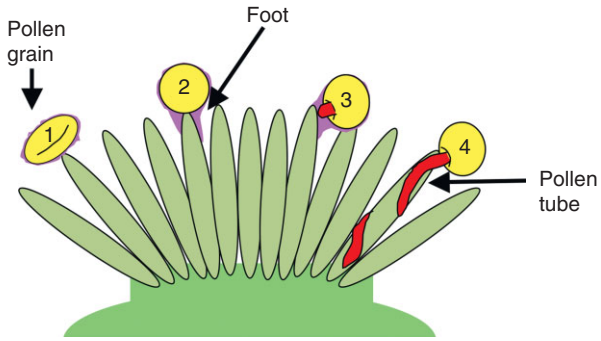

FIGURE 2 | Diagram of early events during pollen tube growth in an *Arabidopsis* pistil. (1) Desiccated pollen grain (oval shaped) adhering to a stigmatic papillar cell. (2) Pollen coat 'foot formation' (pink area between the pollen grain and the stigmatic papillar cell) after completion of hydration (pollen grain is now round shaped). (3) Pollen tube (red) germination, and (4) pollen tube growth toward the style.

review for additional details on this topic.[11] Once the pollen has adhered to the stigma, its journey is guided and regulated by the pistil at each step, including pollen hydration, germination, growth through the style and transmitting tract, guidance to the micropyle, and finally, interaction with the female gametophyte (Figures 1 and 2).

INITIATION OF THE JOURNEY: REGULATION OF POLLEN HYDRATION AND TUBE GERMINATION

Pollen Hydration

Hydration is the first step in pollen germination and, rather than being a passive process, is highly regulated (Figure 2). The pollen and stigma components control the water flow to pollen from the stigma and allow only the appropriate pollen to hydrate. Pollen hydration is also beyond the scope of this review, and we refer to a recent review for additional details on this topic.[11]

Pollen Tube Germination

Physiological activation of the pollen grain triggered after hydration ultimately results in protrusion from the grain as a tube; this is termed pollen germination (Figure 2). Pollen germination is rapid *in vivo*; for example, in *Arabidopsis*, pollen germination occurs in less than 30 min.[15] In plants with wet stigmas (e.g., tobacco), the exudates released by the secretory zone of the stigma promote pollen germination and tube penetration of the stigma.[16,17] Consistent with these observations, tobacco pistils lacking the stigmatic secretory zone (caused by the expression of the cytotoxic barnase gene from a stigma-specific promoter, *STIG1*) are female sterile as most pollen grains are unable to germinate on the ablated stigma.[18] Using these stigmaless pistils, it was later shown that lipids in the wet stigma exudate are the essential components needed for pollen tubes to penetrate the stigma.[19] In plants with dry stigmas (e.g., *Arabidopsis*), the pollen coat fulfills the role of the exudates in wet stigmas. Similar to wet stigmas, lipids in the pollen coat are required for pollen germination, perhaps controlling water flow to pollen from the stigma.[19,20]

Live imaging of Ca^{2+} dynamics in *Arabidopsis* also showed that in the pollen grain, $[Ca^{2+}]_{cyt}$ increases at the potential germination site soon after hydration and remains elevated until tube emergence.[21] These results are consistent with earlier studies that demonstrated an influx of calcium and physiological activation of hydrated pollen grains.[22] The source for the influx of calcium into the pollen grain is unknown; it may come from the cytoplasm or the cell wall of the stigma papilla cell. Pollen grain physiological activation is marked by distinct cytoplasmic reorganization[23,24] resulting in the formation of a cytoplasmic gradient of Ca^{2+} beneath the pollen tube emergence site.[23]

In addition to providing water for pollen hydration, the pistil likely produces factors that promote pollen germination. Indeed, despite the water in pollen growth medium (PGM), *in vitro* pollen germination is not as rapid as *in vivo* germination.[15] Factors that stimulate pollen germination have been found in several species; however, their chemical properties indicate that different molecules are functioning in different species. For example, an unidentified water-, ether-, and methanol-soluble factor in *Chrysanthemum* floral organs significantly increased *in vitro* pollen germination.[25] In tobacco and tomato, an unidentified heat-, acid-, base-, Dithiothreitol (DTT)-, and protease-resistant component from styles, STIL (STyle Interactor for *Lycopersicum esculentum* protein receptor kinases; also see below), increased pollen tube lengths of *in vitro* germinated pollen in a dose-dependent manner.[26] A flavonol (kaempferol) in petunia stigma extracts was shown to stimulate petunia pollen germination *in vitro* and biochemically complement germination and growth defects in petunia pollen lacking chalcone synthase (*CHS*), the enzyme that catalyzes the first step in flavonoid biosynthesis pathway.[27] It is unclear whether flavonoids stimulate pollen germination in all plants. For example, pollen of the flavonol-deficient maize *chs* mutant germinates readily *in vitro* and

in vivo without addition of flavonols.[28] Additionally, null mutation in the single *CHS* gene in *Arabidopsis* does not affect pollen germination, growth, or its ability to fertilize ovules.[29,30]

After germination, the pollen tube must grow in the correct direction and pollen tubes almost always grow down toward the style, indicating the existence of stigma and style factors that direct pollen tube growth toward the ovary (Figure 2). One candidate that may mediate initial guidance of pollen tubes is chemocyanin, a small basic protein first isolated from lily stigmas; indeed, chemocyanins attract and reorient lily pollen tube growth *in vitro*.[31] Wild-type pollen tubes growing on stigmas overexpressing the *Arabidopsis* chemocyanin homolog make numerous turns around the stigma before growing toward the style, in striking contrast to the targeted downward pollen tube growth observed on wild-type stigmas.[32]

CONTINUATION OF THE JOURNEY: SIGNALS THAT FACILITATE POLLEN TUBE GROWTH THROUGH STYLE AND TRANSMITTING TISSUES

Pollen Tube Growth in the Style

Successful germination and growth of the pollen tube through the stigma is followed by entry into the style tissue. Besides serving as a conduit for the pollen tube to reach the transmitting tissue and ovary, style tissues also enable the pollen tube to become competent to perceive guidance signals from the female gametophyte. This conclusion is based on increased ovule targeting efficiency of pollen tubes in *in vitro* assays.[33,34] How does the pistil modify the pollen tube to render it competent to find the ovules? Functional characterization of genes whose expression in pollen tubes are dramatically altered after pollen tube growth through the stigma and style tissues may address this question.[35,36] Another approach is to identify pistil factors that alter pollen tube function. Examination of LePRK1 and LePRK2, tomato plasma membrane localized leucine rich repeat (LRR) receptor-like kinases specifically expressed in pollen grains and tubes,[37] showed that these kinases form a complex[38] that is stable during pollen tube germination *in vitro* and disassociates if tomato or tobacco stylar extract is added.[37,38] Complex dissociation is likely triggered by dephosphorylation of LePRK2, which remains phosphorylated in pollen and *in vitro* germinated grains.[38] LePRK2 dephosphorylation is mediated by a heat-, acid-, base-, DTT-, and protease-resistant component from tobacco style exudates.[26] Downregulation of LePRK2 in pollen tubes leads to reductions of pollen tube germination and tube growth rate, indicating that LePRK2 is required for proper pollen tube functions.[39] Together, these results demonstrate that style components influence pollen tube function by regulating the posttranslational status of LePRK in pollen tubes.

Pollen Tube Growth in the Transmitting Tract

Pollen tubes grow past the style and eventually penetrate the transmitting tract tissue, where a nutrient-rich ECM supports pollen tube growth. In *Arabidopsis*, disruption of the *No Transmitting Tract* (*NTT*) gene, which encodes a C2H2/C2HC zinc finger transcription factor and is expressed specifically in transmitting tissue, affects either the production or composition of ECM.[40] In *ntt* mutants, because pollen tubes grow more slowly and/or terminate prematurely, seed set occurs only in the upper half of the ovary, demonstrating that transmitting tissue is important for facilitating pollen tube growth over a long distance and enhancing the fertilization efficiency of ovules located in the lower half of the ovary.[40] Similarly, loss of three closely related *HECATE* genes (*HEC1-3*), which encode putative basic helix-loop-helix (bHLH) transcription factors, causes defects in septum, transmitting tract and stigma development, and impaired pollen tube growth.[41]

Transmitting tract-expressed proteins important for pollen tube growth are beginning to be identified. In combination with pectin, a 9 kDa lipid transfer protein has been shown to mediate adhesion of pollen tubes to the stylar matrix in lily.[42,43] Two *Arabidopsis* Auxin Response Factors, *ARF6* and *ARF8*, regulate gynoecium and stamen development in immature flowers. Wild-type pollen grew poorly in *arf6 arf8* gynoecia, correlating with ARF6 and ARF8 expression in style and transmitting tract.[44] An important advance in identifying transmitting tract-expressed genes was made recently. By comparing the transcriptional profiles of ovaries isolated from wild-type *Arabidopsis* and from transgenic plants in which the transmitting tract was specifically ablated by the expression of a cytotoxin, a set of 34 genes were found to be expressed specifically in *Arabidopsis* transmitting tract.[45] This set of genes included several putative secreted proteins that may mediate signaling between the pollen tube and the transmitting tract.[45]

A Role for Arabinogalactan Proteins in Pollen Tube Growth

Arabinogalactan proteins (AGPs) expressed in the pistils are transported to growing pollen tubes where they

control signaling and trafficking processes. Phenylglycosides, which specifically bind and precipitate AGPs, inhibit pollen tube growth when injected into transmitting tissues.[46] Furthermore, the transmitting tract-specific (TTS) glycoproteins TTS1 and TTS2 from *Nicotiana tabacum* and *NaTTS* from *N. alata* are localized to the transmitting tract ECM and stimulate pollen tube growth *in vitro*.[47,48] Although potential homologs of these proteins exist in *Arabidopsis*, their role in pollen tube growth is yet to be determined.[47] Transgenic tobacco plants expressing antisense *TTS1* and *TTS2* mRNA are sterile.[48,49] These TTS proteins display a gradient of increasing glycosylation that correlates with the direction of pollen tube growth; both *in vitro* and *in vivo* studies indicate that the TTS proteins[49] and another AGP, NaPRP5,[50] are incorporated into pollen tubes where they are deglycosylated.

Uncommon Amino Acids Have Unusual Roles in Pollen Tube Growth

Two studies have shown that uncommon amino acids have a role in mediating pollen tube–pistil interactions in the style and transmitting tract. Characterization of *pollen on pistil 2* (*pop2*), a mutant that is defective in a gene encoding a gamma-aminobutyric acid (GABA) transaminase, identified a role for GABA in pollen tube growth and guidance in *Arabidopsis*.[51] POP2 activity degrades GABA and likely plays a role in generating a GABA gradient in the pistils, with the maximal GABA concentration in the ovule micropyle.[51] Exogenous GABA also influences pollen tube growth *in vitro*, stimulating pollen tube growth at lower concentrations and inhibiting tube elongation at higher concentrations.[51] High concentrations of GABA are also prohibitive to pollen tube elongation *in vivo*. For example, *pop2* mutants have very high GABA levels in their pistils, resulting in sterility.[51] Recently, *in vivo* pollen tube growth in *pop2-1* pistils was reported. In self-pollinated *pop2-1/pop2-1* pistils, the mutant tubes do not traverse the entire length of the *pop2-1* pistil and fail to elongate beyond the middle point of the transmitting tract.[52] As reported for other *pop2-1* reproductive defects,[51] this pollen tube elongation defect is also self-sterile in nature; the defect manifests only when mutant pollen tubes elongated through a mutant pistil but not with wild-type pollen on a *pop2-1/pop2-1* pistil or *vice versa*. Thus, the high concentrations of GABA (as previously reported in *pop2-1/pop2-1* transmitting tract[51]) are also prohibitive to pollen tube elongation *in vivo*. These results also indicate that the low seed set in a self-pollinated *pop2-1/pop2-1* pistil is due to the combined effects of pollen tube elongation and guidance defects.[52]

Another recent study highlighted the role of the rare amino acid D-serine (D-ser) in facilitating normal pollen tube growth in the style, transmitting tract, and ovules.[53] D-ser activates glutamate receptors (GLRs) in the apical region of pollen tubes, allowing Ca^{2+} influx into the cytoplasm and affecting both Ca^{2+} intensity and oscillation amplitudes in pollen tubes. These conclusions were derived from two sets of experiments. First, pollen tube growth was disrupted in *Arabidopsis* pistils defective in serine racemase (encoded by *SR1*, *At4g11640*) activity, which is required to generate D-ser from the physiologically inactive L-ser. *SR1* is expressed in the style, transmitting tract, and ovules, with maximal expression in the ovule micropyle.[53] Second, D-ser was the most active agonist of *GLR*s in tobacco and *Arabidopsis* pollen tubes. Additionally, it was shown that *in vitro*-grown pollen tubes with lesions in *Atglr1.2* *(At5g48400)* and *Atglr3.7* *(At2g32400)* grew slower than wild type or contained abnormally deformed tips. Finally, it was shown that *Arabidopsis* GLR activity is involved in Ca^{2+} signaling in the pollen tube by controlling $[Ca^{2+}]_{cyt}$ through Ca^{2+} influxes in pollen tubes.[53] Taken together, these results demonstrate that pistil-generated D-ser is the key agonist of GLR, which regulates the influx and intracellular dynamics of calcium in pollen tubes.

How Do Pollen Tubes Emerge Into the Ovary From the Transmitting Tract?

When the pollen tube emerges from the transmitting tract, it forays into the ovary chamber of the pistil. In the ovary, the pollen tube grows on the septum, to which the ovules are attached, and begins to approach the ovule micropyle with remarkable precision (Figure 3). It is notable that many aspects of pollen tube growth in the transmitting tract remain unexplored. First, how does a pollen tube makes the transition from growing in the transmitting tract to emerging onto the septum? Second, how does the pollen tube burrow its way out of the transmitting tract to the septum? Are cell wall-digesting enzymes involved? If so, how are the activities of these enzymes regulated such that they are active in the pollen tube at the time of emergence but not during its growth in the transmitting tract? It appears that pollen tube emergence is associated with localized degradation of the cuticle that lies above the transmitting tract.[54] Availability of sensitive, microscopic assays that can monitor pollen tube growth in a septum[51,55] should facilitate identification of mutants in which pollen tubes germinate, elongate, and reach the bottom of the pistil but continue to remain in the transmitting tract and fail to emerge into the ovary.

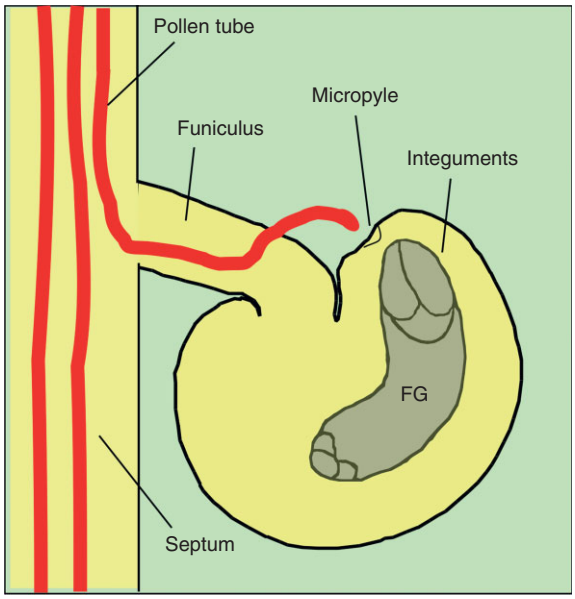

FIGURE 3 | Diagram of pollen tube growth on the septum and ovule of an *Arabidopsis* pistil. Several pollen tubes (red) emerge from the transmitting tract and traverse the septum. However, typically only one pollen tube approaches the funiculus and then targets the ovule micropyle. FG, female gametophyte.

NEXT STEP OF THE JOURNEY: POLLEN TUBE GUIDANCE TO THE OVULE MICROPYLE

Do Pollen Tubes Show a Spatial Preference in Ovule Targeting?

After the pollen tube emerges from the transmitting tract, it migrates on the septum, grows on the surface of the funiculus of an ovule, enters the ovule micropyle, and completes double fertilization in the female gametophyte (Figure 3). One intriguing question is whether pollen tubes show a preference for the apical-most ovule (i.e., is the ovule closest to the stigma is targeted by a pollen tube first before the next ovule in the pistil is approached by the next pollen tube?). In profusely pollinated *Arabidopsis* pistils, pollen tubes do reach the ovules in a temporal series, with the ovules in the apical portion of the pistil receiving first and those in the basal portion of the pistil last.[56,57] However, examination of seed distribution in minimally pollinated wild-type pistils showed a different result.[40] The pollinated pistils were divided into 10 parts starting from the top (0–10 percentile) to the bottom (90–100 percentile) of the ovary chamber, and seed distribution was scored in each segment.[40] In wild type, 62% of the seeds were found in the bottom half of the silique and only 5% were found in the top fifth. These results suggest that there is not a pollen tube preference for fertilization of the apical-most ovules. This decreased preference could be caused by the less-accessible location of apical ovules in *Arabidopsis* (in the shoulder of a pistil and above the transmitting tract; Figure 1(a)). Therefore, a directed time-lapse analysis experiment involving minimally pollinated pistils, using already developed assays,[58] will be essential to convincingly determine the intricacies of ovule preference.

Is There a Role for Sporophytic Tissues in Funicular Guidance of Pollen Tubes to the Ovule?

The pollen tube grows across tissues of the diploid sporophyte for most of its journey. Are these tissues a passive substrate? or Do they play an active role? Specifically, does the funiculus help guide the pollen tube to the ovule? To address this, details of *Arabidopsis* pollen tube growth were examined using confocal and scanning electron microscopy in the septum[51] and funiculus.[59] Although only one pollen tube associates with each ovule, many pollen tubes traverse the septum, indicating that more pollen tubes emerge from the transmitting tract than there are ovules in the ovary.[51] In *Arabidopsis magatama* (*maa*) mutants,[59] the two polar nuclei of the embryo sac fail to fuse, and as a result, a central cell is not formed. In these mutants, the pollen tubes adhere and grow normally on the funiculus but fail to enter the micropyle. These observations prompted the authors to examine the pollen tube path in wild-type ovules; they found no stereotypical path that pollen tubes follow on a funiculus. Instead, the tubes elongate along a boundary of cell files on the funiculus in the proximodistal direction, and when they are within 10 μm of the micropyle, most of the pollen tubes abruptly turn toward the micropyle.[59] Thus, the final stages of pollen tube growth can be divided into two distinct phases: (1) funicular guidance, in which pollen tubes adhere to and grow up the funiculus; this phase is likely mediated by diploid tissues of the ovule and (2) micropylar guidance, mediated by signals from the female gametophyte that guide the pollen tube into the micropyle.[59]

Consistent with this, an extreme pollen tube guidance phenotype occurs in mutants with severe lesions in ovule development, including *bel1*,[60] *sin1*,[61] and *tso1*[62] mutants in *Arabidopsis* and aminocyclopropane-l-carboxylate (ACC) oxidase mutants in tobacco.[63] Because these mutants disrupt both haploid and diploid tissues, it is unclear whether female sporophytic tissues act in funicular guidance of pollen tubes to the ovule micropyle. Evidence in support of a guidance role for diploid ovule cells came

from other studies; female sporophytic *Arabidopsis* mutants *ino*[64] and *pop2*[51] have apparently normal female gametophytes, but pollen tubes exhibit aberrant behavior in the funiculus of the mutant ovules. In maize ovules defective in diSUMO-like protein (*ZmDSUL*), which contain apparently normal sporophytic ovule tissues and disintegrated embryo sacs,[65] pollen tubes migrate normally and arrive within 100 μm from the micropyle but do not enter the ovule.[66] These results suggest that pollen tube guidance to ovules (funicular guidance), with the exception of 100 μm from the micropyle (micropylar guidance), is controlled by sporophytic tissues.[66]

Characterization of pollen tube guidance defects in *pop2* mutants also demonstrated a role of sporophytic tissues in funicular pollen tube guidance.[51] Reciprocal crosses between plants heterozygous for the *pop2* mutation and homozygous for the wild-type or mutant alleles showed that defective sporophytic tissues always associated with pollen tube guidance defects. For example, pollen tube guidance defects were observed only when *pop2* mutant pollen tubes grew toward *pop2* homozygous mutant ovules but not to the heterozyous ovules. These results indicated that the genotype of the sporophytic ovule cells (by inference, the abundance of GABA in the sporophytic, diploid ovule tissues) that surround the female gametophyte, rather than that of the female gametophyte, causes funicular guidance defects in *pop2* mutant.[51]

Female Gametophyte and Micropylar Guidance of the Pollen Tube to the Ovule

What is the origin of the micropylar signals that guide the pollen tube on the last part of its journey? Strong evidence that the female gametophyte is the sole source of these signals came from a study using ovules with aberrant female gametophytes surrounded by normal sporophytic tissues.[67] Pistils in which half the ovules contained apparently normal diploid tissues yet lacked a haploid female gametophyte were generated by using reciprocal chromosomal translocations. When these pistils were pollinated, pollen tubes exhibited normal micropylar guidance only to the ovules with normal female gametophyte but not those with aberrant female gametophytes.[67]

Micropylar guidance signals originate at least in part from synergid cells contained within the female gametophyte. For example, pollen tubes do not enter ovules in which synergid cells were either laser ablated[68] or defective with abnormal filiform apparatus, a membrane-rich region at the micropylar end of the synergid through which a pollen tube enters the female gametophyte[69]; also see below). Recently, cysteine-rich polypeptides (CRPs), a subgroup of defensin-like proteins referred to as LUREs, were identified from *Torenia fournieri* synergid cells and shown to be attractants that can mediate micropylar guidance of pollen tubes.[70] Importantly, recombinant LUREs have chemoattractant activity *in vitro* toward competent pollen tubes of their own species.[70] Finally, administration into synergid cells of morpholino antisense oligomers against the LURE genes abolished pollen tube attraction, indicating that these LUREs act *in vivo* as pollen tube attractants.[70] Recently, a CRP from *T. concolor* was shown to function as a chemoattractant in a concentration-dependent and species-specific manner.[71]

Progress has also been made in identifying molecular mechanisms that mediate micropylar guidance in other plants. A maize protein, ZmEA1, which is exclusively expressed in the egg and synergids of unfertilized female gametophytes, acts in regulating micropylar guidance.[72] *ZmEA1* encodes a 94 amino acid transmembrane protein. Maize plants expressing *ZmEA1* RNAi or antisense constructs produced significantly fewer seeds than wild type, and wild-type pollen tubes failed to enter mutant ovules. The loss of guidance in *ZmEA1* RNAi or antisense plants[72] is similar to the phenotype in plants that lacked female gametophytes,[66] indicating that ZmEA1 might represent the sole pollen tube attractant in maize.[73] A ZmEA1–green fluorescent protein (GFP) fusion protein localizes first to the filliform apparatus in the synergid cells and then appears in the cell walls of neighboring nucellar cells, suggesting that ZmEA1 may be released from the synergid cells and mediate short-range micropylar pollen tube guidance.[72] The accumulation in the nucellar cells is significant considering that in the grass family, these cells enclose the female gametophyte and form another barrier to pollen tube entry. These protein localization studies raise the possibility that ZmEA1 protein is a chemoattractant. Indeed, recombinant ZmEA1 polypeptide directly attracts maize pollen tubes at low concentrations (<10 μM) *in vitro*.[74] So far, EA1 and LUREs are the only chemoattractants with a demonstrated role in mediating short-range, micropylar guidance of pollen tubes to ovules.

In *Arabidopsis*, insights into short-range micropylar guidance signals were obtained using an *in vitro* pollen tube guidance assay.[34] It was shown that *Arabidopsis* ovules emit diffusible, heat-labile, developmentally regulated, species-specific attractants whose molecular weight could be ≤ 85 kDa.[34] The identity of these chemoattractants in *Arabidopsis* remains unknown; however, the *myb98* mutant offers an important opportunity to identify them. The

myb98 synergid has a defective filliform apparatus, and wild-type pollen tubes fail to enter *myb98* ovules.[69] The *MYB98* gene is expressed predominantly in the synergid cells and encodes an R2R3-MYB transcription factor. MYB98 may act indirectly in guidance, possibly by either modulating filiform apparatus development to enhance the secretion of chemoattractant or regulating the synthesis of a pollen tube attractant. Consistent with either of these possibilities, several genes that are directly regulated by the *MYB98* transcription factor were identified.[75] The protein products of these genes localize to the filiform apparatus[75] and diffuse out into the micropylar region of the ovule,[76] similar to the localization and diffusion pattern of the maize pollen tube attractant, ZmEA1 (Ref 72; also see above).

Some *Arabidopsis une* mutants contain apparently normal ovules but exhibit micropylar guidance defects[77]; characterization of these mutants and mutants in recently identified synergid-specific genes[78–80] should offer additional insights into the micropylar guidance of pollen tubes to ovules. Besides synergid cells, in *Arabidopsis*, the central cell[81] and the egg cell[82] may also function in micropylar guidance of pollen tubes. For example, lesions in *Central Cell Guidance* (*CCG*) result in micropylar guidance defects.[81] *CCG* is expressed in the central cell and encodes a nuclear protein containing a domain that is functionally interchangeable with that of transcription factor IIB (TFIIB) in yeast. It is proposed that CCG may act as a transcriptional regulator of downstream genes that regulate pollen tube guidance either directly or in conjunction with the synergids or egg cell.[81] Consistent with the proposed role for the central cell in pollen tube guidance, *maa* mutants with defective polar nucleus fusion are defective in micropylar guidance of pollen tubes.[59] Similarly, loss of GEX3, a plasma membrane protein that is expressed in the egg cell, also results in defective micropylar guidance, highlighting the role for the egg cell in micropylar guidance.[82]

CULMINATION OF THE JOURNEY: POLLEN TUBE INTERACTIONS WITH THE FEMALE GAMETOPHYTE

Order of Events during Interactions Between the Pollen Tube and the Female Gametophyte

After entering the ovule micropyle, the pollen tube reaches one of the synergid cells, stops growing, and bursts, releasing the two sperm cells to complete double fertilization. In many species, including

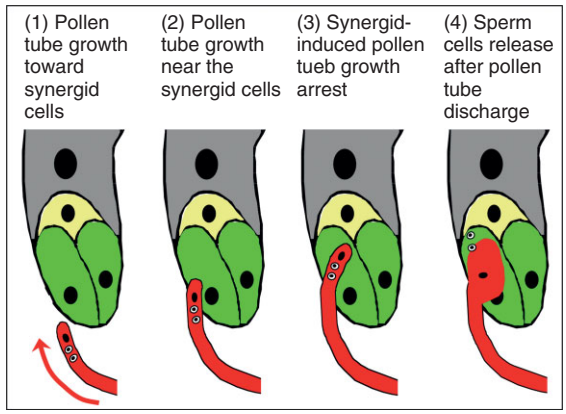

FIGURE 4 | Temporal sequence of pollen tube–female gametophyte interactions within an ovule during *Arabidopsis* reproduction (left to right). The hallmark event at each step is indicated and only the micropylar region of an ovule is shown at each step. Egg cell is shaded in yellow, two synergid cells and central cell are shaded in green and grey, respectively.

Arabidopsis, the synergid cell that receives the pollen tube degenerates either at the time of pollen tube entry into the ovule or shortly thereafter.[83] Using confocal laser scanning microscopy, light microscopy, transmission electron microscopy, and real-time imaging, the temporal sequence of events in pollen tube–female gametophyte interactions was examined in *Arabidopsis*[56] and shown to occur in the following order: (1) pollen tube migration to a synergid cell, (2) pollen tube growth near the synergid cell, (3) pollen tube growth arrest in the synergid cell, (4) degeneration of the receptive synergid cell, and (5) pollen tube burst and release of the two sperm cells for double fertilization (Figure 4). In *T. fournieri*, synergid degeneration occurred 0.6 ± 0.6 seconds after the start of discharge.[84] Therefore, the order of the last two events are either variable in different species or the techniques used in *Arabidopsis* are not as sensitive as those used in *T. fournieri*. Identification of mutants defective in this process should help confirm the temporal relationship between synergid degeneration and pollen tube discharge in *Arabidopsis*.

Consistent with this proposed sequence of events, mutants defective in pollen tube growth arrest in the synergid cell [*sirene* (*srn*), *abstinence by mutual consent* (*amc*), and *lorelei* (*lre*); also see below], were also defective in downstream events such as pollen tube discharge in the synergid cell and synergid cell degeneration (Refs 57, 58, 85; Figure 5). Similarly, although pollen tubes entered, reached, and arrested growth in the *vdd* mutant female gametophytes and synergid degeneration occurred, the mutant ovules were not fertilized,[86] providing further support to the proposed order of events inside the ovule (Figure 5).

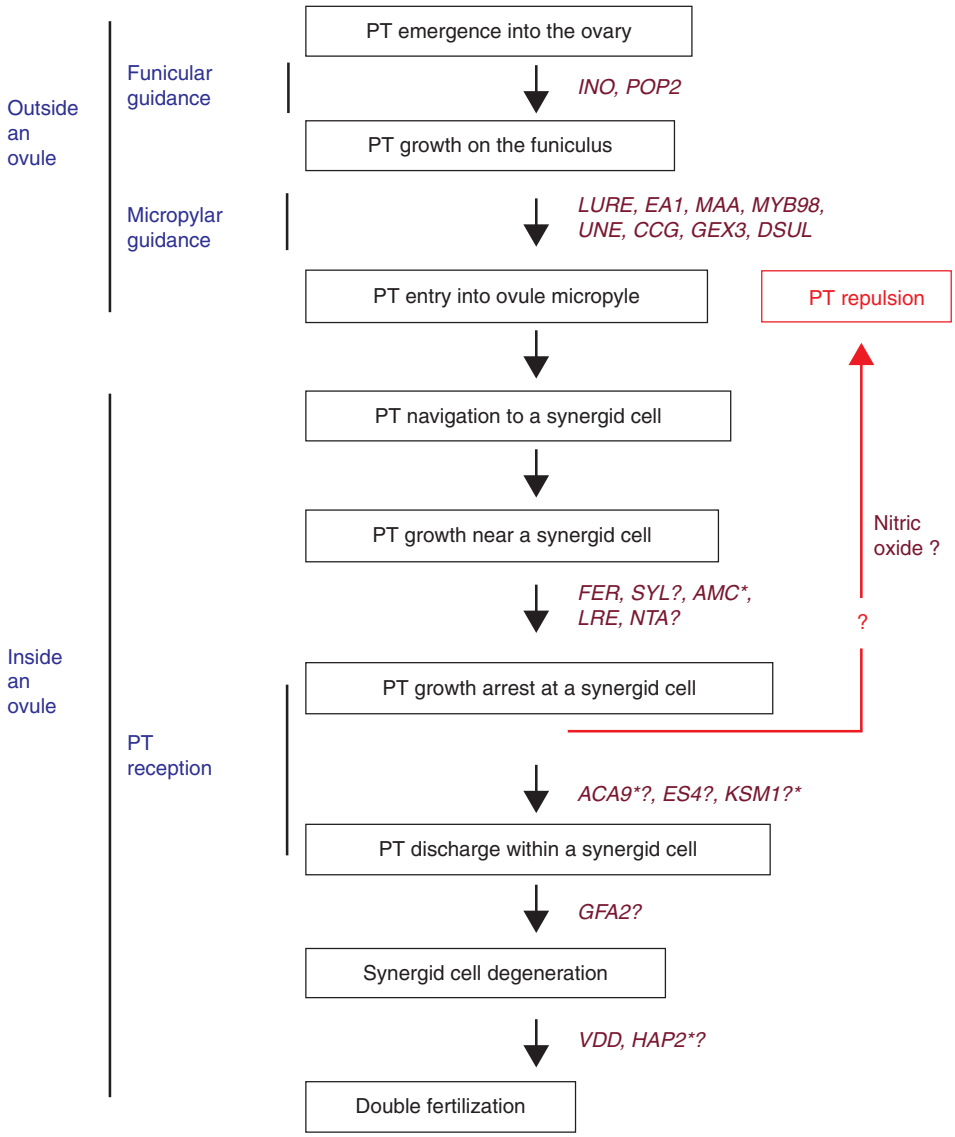

FIGURE 5 | Major events during pollen tube–female gametophyte interactions in plant reproduction. The genes (based on mutant analysis) that mediate indicated steps in the pollen tube–female gametophyte interactions. Question marks indicate that the placement of the gene in that position is based on data that tested only in some of the steps shown in this figure. Genes marked with an asterisk are known to function in pollen tubes. PT, pollen tube.

Recently, mutants disrupted in pollen tube–female gametophyte interactions have been reported; more extensive phenotypic characterizations of these mutants will provide additional insights into the order of these key events. For example, synergid degeneration fails to initiate in *gfa2* female gametophytic mutants,[87] but events upstream of this defective step remain uncharacterized (Figure 5). Similarly, some *aca9* pollen tubes arrest growth in the ovule but do not discharge and release the sperm cells,[88] indicating that pollen tube discharge is downstream of cessation of pollen tube growth in the synergid cell. However, in such ovules showing mutant pollen tube behavior, whether the synergid cell has degenerated is not known (Figure 5). Finally, *hap2* (*gcs1*) pollen tubes arrest growth and discharge the sperm cells but do not complete fertilization.[89,90] However, in this mutant, the status of synergid cell degeneration is also not known (Figure 5).

Mutants Defective in Pollen Tube Growth Arrest in the Synergid Cells of the Female Gametophyte

Several *Arabidopsis* genes involved in regulating pollen tube growth arrest in the synergid cells

of the female gametophyte have been identified. *FERONIA/SIRENE (FER/SRN)*, a receptor-like serine/threonine kinase, is essential for pollen tube growth arrest in synergids; after arriving in a *fer/srn* female gametophyte, the pollen tube fails to arrest its growth, grows excessively around the synergids, and fails to discharge the sperm cells.[7,58,91] A FER:GFP fusion protein localizes to the plasma membrane and is enriched in the filiform apparatus, a membrane-rich region at the micropylar end of the synergid.[91] Thus, *fer* is referred to as defective in 'pollen tube reception', a term that collectively refers to the pollen tube growth arrest and discharge steps in the female gametophyte (Ref 7; Figure 4). However, since the *aca9* mutant phenotype demonstrated that pollen tube growth arrest is separable from pollen tube discharge,[88] we propose that mutants that exhibit a 'feronia' phenotype (i.e., excessive growth of pollen tubes in the synergid cell) be specifically termed as defective in pollen tube growth arrest (Figures 4 and 5).

Other mutants that disrupt pollen tube growth arrest include *lre* and *scylla* (*syl*).[57,92,93] *LRE* encodes a putative glycosylphosphatidylinositol (GPI)-anchored membrane protein,[57,92] and the molecular identity of SYL is unknown.[93] Pollen tube reception also requires functions of the pollen tube; in the self-sterile *amc* mutants, pollen tube reception fails only when an *amc* female gametophyte interacts with an *amc* pollen tube. *AMC* encodes a peroxin that is critical for importing proteins into peroxisomes.[85] A fully differentiated filiform apparatus is not essential for pollen tube growth arrest, as filiform apparatus development is defective in *myb98* ovules, yet the small percentage of pollen tubes that do enter the ovules (perhaps randomly or as a result of minimal chemoattractant) arrests growth normally within these mutant ovules.[69]

Role of FERONIA-Dependent Signaling in Pollen Tube Growth Arrest in the Synergid Cell

Although the molecular mechanisms by which AMC and LRE function to regulate pollen tube growth arrest are unknown, more information is known about how FER mediates pollen tube growth arrest. It is postulated that FER localizes to the synergid cell membrane, and the extracellular domain of FER either binds to a ligand from the pollen tube[91] or responds to a synergid maturation signal from the other female gametophyte cells,[92,93] leading to autophosphorylation of its cytoplasmic kinase domain and initiation of an unknown signal transduction cascade in the synergid cell that leads to the induction of pollen tube growth arrest (Ref 91; Figure 6).

NORTIA Functions Downstream of FERONIA in the Synergid Cell and Induces Pollen Tube Growth Arrest

In a search for additional components of the FER signaling pathway, Kessler et al.[94] recently reported on the female gametophyte-specific *nortia* (*nta*) mutant, which shows a failure of pollen tube growth arrest similar to that observed in *fer* female gametophytes. *NTA* encodes a 7-transmembrane receptor protein of the powdery Mildew Locus of Resistance (MLO) family. Before pollen tube arrival at the synergid cell, NTA:GFP is distributed throughout the synergid cell. However, upon pollen tube arrival, NTA:GFP becomes localized in the micropylar pole of the synergid cell, which includes the filiform apparatus.[94] Interestingly, in *fer* female gametophytes, NTA:GFP remains distributed throughout the synergid cell even after pollen tube arrival, indicating that NTA redistribution is under the direct or indirect control of FER.[94]

Insights Into NORTIA Function in the FERONIA Signaling Pathway From Powdery Mildew Invasion of Leaf Epidermal Cells

The pollen tube arrival-dependent redistribution of NTA:GFP in the receptive synergid is strikingly similar to MLO protein redistribution in barley leaf epidermal cells upon infection with powdery mildew (PM). Fluorophore-tagged MLO proteins accumulate in foci at sites where PM fungal hyphae attempt to penetrate the leaf epidermal cell wall.[95] These sites are also where membrane microdomains accumulate to receive vesicles carrying defense compounds and regulatory proteins that are delivered by the host cell to block PM invasion. The accumulated MLO proteins act on syntaxin proteins involved in vesicle transport and neutralize the host cell defense against PM invasion. Consequently, host-expressed MLO proteins are necessary for PM invasion.[96]

The MLO protein interacts with the cytoplasmic calcium sensor calmodulin via a calmodulin-binding site in its carboxy-terminal cytoplasmic tail.[97] Upon fungal hyphal penetration of the host cell, MLO calmodulin-binding activity increases around the penetration sites, indicating that MLO protein activation coincides with increased in intracellular calcium.[95] Since the MLO protein (NTA) is regulated by FER, Kessler et al.[94] investigated potential parallels between pollen tube growth arrest and fungal invasion by examining the role of FER in PM invasion of plant cells. Indeed, *fer/fer* plants were resistant to PM invasion, and introduction of a wild type copy of *FER*

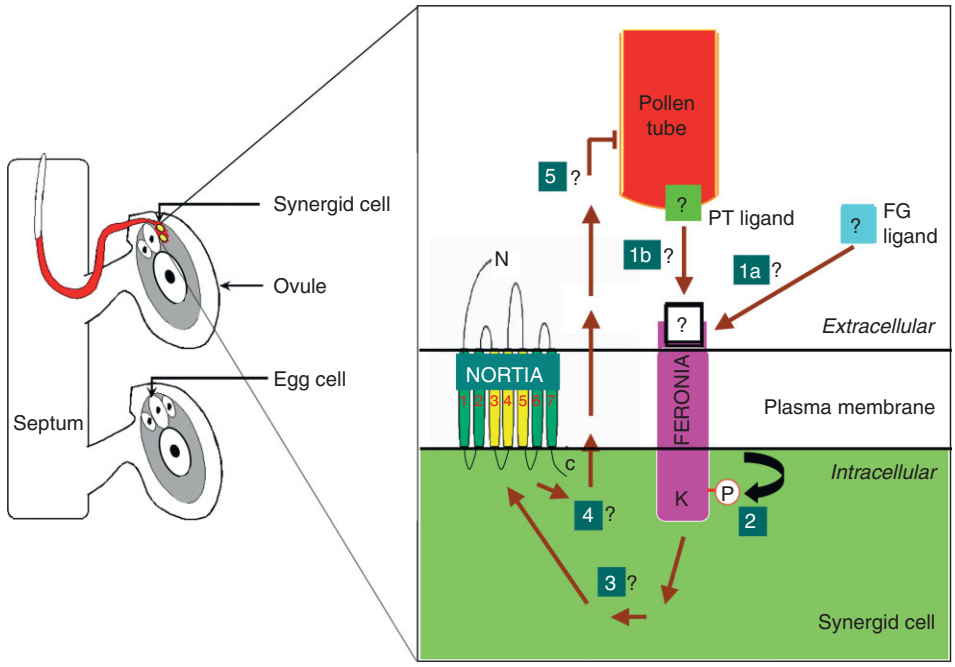

FIGURE 6 | Putative FER signaling pathway in an *Arabidopsis* synergid cell prior to or at the arrival of the pollen tube. (1) Binding of an unknown ligand from either the female gametophyte (FG, 1a) or the pollen tube (PT, 1b) with FER results in (2) autophosphorylation of FER receptor-like kinase, initiating (3) an uncharacterized signaling pathway which perhaps redistributes NTA to pollen tube entry site in the synergid cell, leading to (4) generation of a secreted signal that ultimately (5) culminates in inducing pollen tube reception/growth arrest. Question marks indicate putative steps.

into *fer/fer* plants rendered them susceptible to PM invasion.[94] The conservation of molecular components mediating these two seemingly distinct processes (facilitating fungal invasion into leaf epidermal cells and inducing pollen tube reception prior to entry into synergid cells) has been used to expand the model of FER function in pollen tube growth arrest: upon induction of FER signaling, NTA might accumulate in membrane domains at the site of pollen tube entry, neutralize the vesicle delivery of agents that prevent pollen tube invasion, and enhance delivery of signals to induce pollen tube growth arrest (Ref 98; Figure 6).

Pollen Tube Discharge

After the pollen tube ceases growth in the female gametophyte, it is induced to release the sperm cells (Figure 4). In *T. fournieri*, the discharge from pollen tubes was observed in an *in vitro* assay; the pollen tube explosively discharges its contents at an initial rate of about 12,000 $\mu m^3 s^{-1}$.[84] In *Arabidopsis*, pollen tube discharge has been observed *in vivo*; the pollen tube forms an extension in the synergid cell toward the direction of the central cell and explosively discharges its contents within a minute of reaching the synergid cell.[58] A similar sequence of events during pollen tube discharge was also observed in an *in vitro* assay.[34]

In *Plumbago zeylanica*, which lacks synergids, the egg cell carries out synergid functions and, when the pollen tube reaches the egg cell, a terminal pollen tube aperture forms through which the sperm cells are released.[99]

Signals from the female gametophyte that induce pollen tube rupture are best characterized in maize. A synergid-expressed defensin-like protein, *ZmES4*, interacts with the K+ Shaker channel KZM1, which is localized at the pollen tube tip plasma membrane and causes the pollen tube to rupture.[100] Consistent with its role in the release of sperm cells for fertilization, loss of *ZmES4* resulted in reduced seed set. Importantly, recombinant ZmES4 triggered rapid plasma membrane depolarization and caused *in vitro* grown pollen tube tips to rupture, demonstrating that ZmES4 is necessary and sufficient to induce pollen tube rupture.[100]

Besides KZM1, three other pollen tube-expressed genes in *Arabidopsis* also act in pollen tube tip rupture. *ANXUR1* (*ANX1*) and *ANXUR2* (*ANX2*) are closely related to FER receptor kinase and expressed in pollen. Double mutant (*anx1;anx2*) pollen tubes rupture prematurely in the transmitting tract, indicating that ANX proteins repress pollen tube rupture until after reaching the synergid cell.[101,102] *ACA9* of *Arabidopsis* is a calcium transporter, and

loss of this pump results in pollen tubes that reach the female gametophyte and arrest growth but fail to discharge.[88] It remains to be determined how these three genes function together in pollen tube discharge. Also, the ligands (the functional equivalent of ZmES4) released by the synergid cells in *Arabidopsis* remain to be identified.

AT THE END OF THE POLLEN TUBE JOURNEY: PREPARATION FOR DOUBLE FERTILIZATION

Synergid Cell Degeneration

One of the two synergid cells, typically the one at which the pollen tube has arrived and arrested growth, undergoes programmed cell death. In *Arabidopsis*, synergid degeneration occurs after the pollen tube arrival at the female gametophyte.[56] Thus, synergid degeneration is not required either for pollen tube entry into the micropyle or migration to the female gametophyte.[56] This is supported by observations that the pollen tube arrival in the female gametophyte in *srn*, *amc*, and *lre* mutants is comparable to the wild type, even though synergid degeneration does not occur in these mutants.[57,58,85] However, since synergid degeneration does not occur in these three mutants that are also defective in pollen tube growth arrest, it remains to be determined if synergid degeneration is required for pollen tube growth arrest. One way to determine the temporal relationship between pollen tube growth arrest and synergid degeneration is to characterize synergid degeneration in *aca9* mutants, which fail to discharge sperm cells, and to analyze the pollen tube growth phenotype in *gfa2* mutants, which fail to initiate synergid degeneration (Figure 5).

Double Fertilization

The major steps of double fertilization are proposed to occur in the following order: (1) the release of the two sperm cells, (2) the migration of the sperm cells to the two female gametes, (3) gamete recognition and plasmogamy, (4) karyogamy, and (5) initiation of seed development, which includes reinitiation of the cell cycle, transcription, and translation in the zygote.[10,103,104] During the past few years, molecular genetic analysis of double fertilization in *Arabidopsis* has resulted in several exciting developments, including the identification of a sperm cell-specific and membrane-localized HAP2/GCS1[89,90] that is essential for gamete fusion in higher plants, *Chlamydomonas*, and *Plasmodium*.[105] Details about the function of these proteins and other recent findings related to sperm–egg fusion are beyond the scope of this review, and therefore, we refer the reader to other recent reviews on this topic.[8,106,107]

WARDING OFF ADDITIONAL SUITORS: POLLEN TUBE REPULSION

Multiple Pollen Tubes Typically Do Not Enter an Ovule

Characterization of pollen tube growth near an ovule has typically shown only one pollen tube emerging from the septum, climbing up the funiculus, and entering the micropyle. This is striking considering that many pollen tubes emerge from the transmitting tract and migrate on the septum; yet, only one pollen tube approaches an ovule (Figure 3). Occasionally, multiple pollen tubes approach an ovule in a pistil,[58] especially in the apical region of *Arabidopsis* pistils[51]; however, despite coming close to the micropyle, multiple tubes typically do not enter an ovule. Finally, pollen tubes do not enter fertilized ovules in the *T. fournieri*[33] and *Arabidopsis*[34] *in vitro* assays. On the basis of these results, two nonexclusive reasons could be proposed for prevalence of pollen tube repulsion. First, pollen tube repulsion contributes to preventing genomic conflicts that would arise from the egg cell and central cell being fertilized by genetically distinct sperm.[108] Second, pollen tube repulsion prevents multiple pollen tubes from growing to one ovule; instead, it facilitates fertilization of sibling ovules and increases inclusive fitness, similar to the selfless behavior of haploid female worker bees that help their close relatives.[59] Despite the existence of such prevention mechanisms, multiple tubes do enter an ovule at a low frequency.[109] In maize, heterofertilization results when the egg and central cell are fertilized by different pollen tubes at a frequency of ~2%[110] and in *Arabidopsis* ~1%.[7]

When Does Pollen Tube Repulsion Initiate?

At which stage during the pollen tube–ovule interactions is pollen tube repulsion initiated? Clues to this question come from analysis of female gametophytic mutants that are defective in the final stages of pollen tube growth. On the basis of the analysis of *Arabidopsis* female gametophytic mutants, pollen tube repulsion is initiated only after pollen tube growth arrest in synergid cells (Figure 5); mutant ovules, defective in pollen tube growth arrest, also allow multiple tubes to enter them.[7,57,58,92,93] Importantly, the partial penetrance of the pollen tube growth arrest defects in *lre* female gametophytes was exploited to examine if pollen tube repulsion can be uncoupled from pollen tube growth arrest.

In all four *lre* alleles examined, every incidence of multiple pollen tube entry was observed in an ovule that was also defective in pollen tube growth arrest; an instance of multiple tubes approaching an ovule with normal pollen tube growth arrest was never observed.[57] Consistent with these results, mutants defective in earlier steps, such as pollen tube entry into the ovule (*maa* and *myb98*; Figure 5), were defective in preventing multiple pollen tubes from entering the mutant ovule.[59,69] To accurately determine the temporal relationship between pollen tube repulsion and the final stages of pollen tube growth, pollen tube repulsion needs to be examined in additional mutants (such as *aca9*, *gfa2*, and *vdd*) that are defective in a step downstream of pollen tube growth arrest (Figure 5).

Origin of Pollen Tube Repulsion Signals

The exclusion of additional pollen tubes after one tube has entered the ovule could be due to termination of ovule attractant release (passive model) or, alternatively, generation of a repulsion signal to repel other pollen tubes (active model). Examination of pollen tube–ovule interactions in *Arabidopsis in vitro* assays lends support for an active model of pollen tube repulsion.[34] First, repulsion of late-arriving tubes initiates rapidly (sometimes within 10 min after the first tube entered the ovule), a time frame likely too short for cessation of attraction signal(s) to have a repulsive effect.[34,57] Second, unsuccessful tubes actively approached and then abruptly altered their path near the micropyle of the ovule that had been targeted by another pollen tube.[34,57]

Pollen tube repulsion signals could be derived either from ovules or pollen tubes. In the case of ovule-based pollen tube repulsion signaling, sporophytic cells in an ovule are one possible source of repulsive signals.[51,111] The female gametophyte also has a role in pollen tube repulsion as evident from defective pollen tube repulsion in several female gametophytic mutants.[7,57–59,69,92,93] Alternatively, the female gametophyte may be involved in generating pollen tube repulsion signals. Support for this possibility comes from the observations that female gametophytic mutants that fail to arrest pollen tube growth in the synergid cell are also defective in initiating pollen tube repulsion[7,57,58,92] (Figure 5). Pollen tube repulsion could originate from competing tubes; multiple pollen tubes growing toward *maa* ovules typically grew parallel to each other and on the opposite sides of the funiculus indicating repulsion between pollen tubes.[59] Another possibility for pollen tube-based repulsion signaling is that discharge from a successful pollen tube could release pollen tube repulsion signals. Examining pollen tube repulsion in mutants defective in processes downstream of pollen tube growth arrest could test this possibility (e.g., *aca9*, *gfa2*, *vdd*, and *hap2*; Figure 5).

What is the nature of pollen tube repulsion signals? Nitric oxide is a candidate for mediating pollen tube repulsion based on its ability to induce lily pollen tubes to make sharp, $>90^{\circ}$ turns away from a nitric oxide source.[112] This behavior is strikingly similar to the pollen tube repulsion behavior near the micropyle of ovules that have already been penetrated by a pollen tube.[34] However, a conclusive *in vivo* link between nitric oxide and pollen tube repulsion is yet to be established. Cross-pollination between wild-type and *atnos1* plants (which have decreased levels of nitric oxide and reduced seed set) showed that the *atnos1* mutation in the pistil tissues resulted in abnormal pollen tube guidance.[111] Additionally, diaminofluorescein-2 diacetate (DAF-2DA) fluorescent staining identified specific areas around the unfertilized ovule micropyle to be significant production sites of nitric oxide.[111] However, an exclusive role for nitric oxide in pollen tube repulsion would predict no reduction in fertility (and perhaps an increased rate of seed abortion) and significant production of nitric oxide primarily in the micropyle of an ovule only after it was targeted by a pollen tube; *atnos1* mutants do not exhibit these phenotypes.

CONCLUSIONS

Over the past few years, significant progress has been made in understanding the molecular and genetic processes that underlie pollen–pistil interactions. It is now evident that a complex set of pistil signals is essential for successful guidance of a pollen tube to its target. However, our understanding is far from complete and several questions remain unanswered. For the newly identified pistil signals, the focus will be on understanding the response mechanisms in pollen tubes and how these signals are generated in the pistil tissues. For signaling pathways implicated in pistil tissues (such as FER signaling in synergids), it will be important to not only isolate additional components of these pathways but also identify the ligands (either from pollen tube or from other cells in the female gametophyte) that activate these pathways. For these advances to materialize, a combination of approaches (biochemical, genetic, and genomic) coupled with interdisciplinary methods developed in several plants will be required. Excitingly, in the recent years, significant advances have also been made in generating tools, techniques, and assays to facilitate the characterization of pollen–pistil interactions.

In *Arabidopsis*, several mutant collections,[77,113–116] including those in which the pollen tube is marked,[55] can be rapidly screened for mutant phenotypes using a variety of genetic and microscopic assays to characterize pollen tube growth, guidance, pistil, and female gametophyte development.[51,55,77,117–119] Additionally, several *in vitro* assays have been developed to test various aspects of pollen tube growth[119] and guidance (Refs 34, 120; Movies 1 and 2). As a complementary approach, gene discovery in a variety of tissues[35,36,45,78–80,121–123] can be coupled with reverse genetic analysis to assign roles for several genes, including those that are specifically expressed in individual cell types of the gametophytes,[8,78] in pollen tube growth and guidance. Pioneering biochemical work in other plants is being efficiently complemented with genomic analysis that is beginning to yield rich dividends.[70,73] Additionally, inclusion of RNAi-[73] and morpholino-[70] based analysis are already allowing major discoveries to be genetically tested in these plants. Considering these significant developments, it is not an overstatement to say that pollen tube research is entering an exciting phase.

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