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A Fruitful Journey: Pollen Tube Navigation from Germination to Fertilization

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Abstract

In flowering plants, pollen tubes undergo tip growth to deliver two nonmotile sperm to the ovule where they fuse with an egg and central cell to achieve double fertilization. This extended journey involves rapid growth and changes in gene activity that manage compatible interactions with at least seven different cell types. Nearly half of the genome is expressed in haploid pollen, which facilitates genetic analysis, even of essential genes. These unique attributes make pollen an ideal system with which to study plant cell–cell interactions, tip growth, cell migration, the modulation of cell wall integrity, and gene expression networks. We highlight the signaling systems required for pollen tube navigation and the potential roles of Ca^{2+} signals. The dynamics of pollen development make sexual reproduction highly sensitive to heat stress. Understanding this vulnerability may generate strategies to improve seed crop yields that are under threat from climate change.

Contents

1. INTRODUCTION

Reproduction in flowering plants has evolved around using pollen tube delivery of sperm to female gametes (**Figure 1**). This fruitful journey begins with the determination of the time and place of pollen germination, proceeds as the pollen tube extends through the stigma, the transmitting tissue of the style, and along an ovule funiculus, and finally ends at a synergid, where the tube bursts to release two sperm cells: one to fuse with the egg cell, the other with the central cell. This last step is referred to as double fertilization (recently reviewed in 74). This journey thus involves interactions with at least seven different cell types (111), requiring multiple changes in gene expression and physiology.

While this review focuses on *Arabidopsis thaliana* (*Arabidopsis*), a pollen grain's reproductive journey has evolved to have numerous variations. For example, some plants evolved structural and molecular features to promote self-fertilization, whereas other plant features promote outcrossing through self-incompatibility mechanisms (see **Supplemental Figure 1** for comparative pistil architecture). Still, pollen in hundreds of thousands of flowering plant species continues to evolve unique morphological and physiological adaptations.

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Arabidopsis provides an excellent reference organism with which to genetically dissect the signaling systems important to pollen development and fertilization. Depending on the cutoff thresholds used for reliable detection and quantification, there might be as many as 14,000 to 16,000 protein coding genes expressed in pollen (57). Even a midrange estimate of 15,000 represents nearly half of the *Arabidopsis* genome.

The continued push for a comprehensive analysis of the *Arabidopsis* genome will inevitably include testing the biological function of nearly every gene during reproductive development. In this effort, the easiest test is whether a mutation or transgene shows a distorted segregation when heterozygous plants are self-fertilized or used in reciprocal crosses to the wild type. Calculating a relative transmission efficiency ratio (TEr) (number of mutant events relative to number of control events) provides a standard way to assess the phenotype and make quantitative comparisons to other mutants or growth conditions (see **Figure 2** for examples). A TEr will hopefully become an annotation linked to every mutation in the *Arabidopsis* genome and in other genetically tractable systems.

In vitro growth of pollen also provides an excellent model for the study of tip growth (106) and how it responds to signals (74). A single review cannot cover the immense amount of interesting research in pollen biology. Instead, this review highlights just a few select features and provides a framework for thinking about how and why varieties of pollen do what they do. We first discuss the fundamentals of tip growth, expand on the importance of Ca^{2+} in pollen tube tip growth, and

Transmitting tract: the link between stigma, style, and ovules in a pistil; rich in extracellular matrix; assists in pollen tube growth to the ovary

Double fertilization: a defining feature of flowering plants in which a pair of sperm cells fuse with a pair of female gametes, the egg, and central cell

Tip growth: anisotropic extension of a cellular surface following new membrane deposition

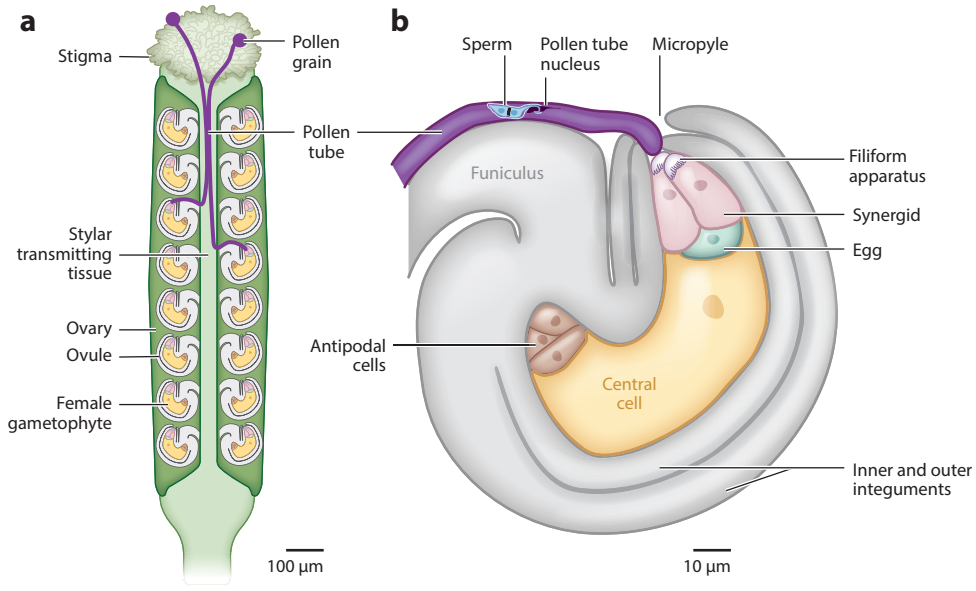

Figure 1

The pollen tube journey from stigma to synergid. (*a*) In *Arabidopsis thaliana*, pollen grains are deposited on the stigma and germinate a pollen tube that enters the transmitting tissue of the style before exiting onto the interior surfaces of the ovary. The pollen tube then enters an ovule and contacts the female gametophyte. (*b*) The pollen tube (male gametophyte) grows along the surface of the funiculus and is attracted into the micropyle by the synergid cells, which have a specialized secretory filiform apparatus at their micropylar pole. The male gametophyte consists of haploid pollen (also termed vegetative cell) and sperm cells, both of which are derived from a single meiotic product. The sperm cells, each with its own distinct plasma membrane, are enclosed in an additional membrane derived from the pollen membrane. In addition to the synergid cells, the female gametophyte consists of an egg, a central cell, and three antipodal cells. The female gametophyte develops within the inner and outer integument cell layers of the ovule. The female gametophyte cells are haploid and descend from a single meiotic product. Central cell development is complete when two haploid nuclei fuse to form a nucleus with two identical sets of haploid chromosomes. Approximate scale bars are given. Comparisons of *Arabidopsis* with tomato and barley (the latter, as a representative monocot) are presented in **Supplemental Figure 1**.

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then elaborate on the major signaling decisions encountered during a pollen grain's journey to the ovule.

2. TIPPING POINT: FUNDAMENTALS OF POLLEN TUBE GROWTH

Tip growth is a special case of anisotropic growth that enables pollen tubes to undergo directional growth to locate and fertilize ovules. Fundamental features of tip growth have been extensively reviewed (18, 42, 48–50, 74, 87, 94, 99, 106, 119, 127, 147). There are many examples of polarized growth in eukaryotes, including fungal hyphae (126) and root hairs and trichomes in some plants (119). Pollen tubes provide examples for some of the fastest growing eukaryotic cells known (119, 129). This rapid growth probably evolved as a consequence of strong selection pressure for the fastest tube to locate an ovule and transmit its genome to the next generation (143). However, with speed comes danger. If a cell grows too fast, it becomes susceptible to bursting if it cannot appropriately balance the turgor forces that drive cellular expansion with the construction of a strong yet flexible cell wall; it may also grow too fast to respond to guidance cues and find an ovule.

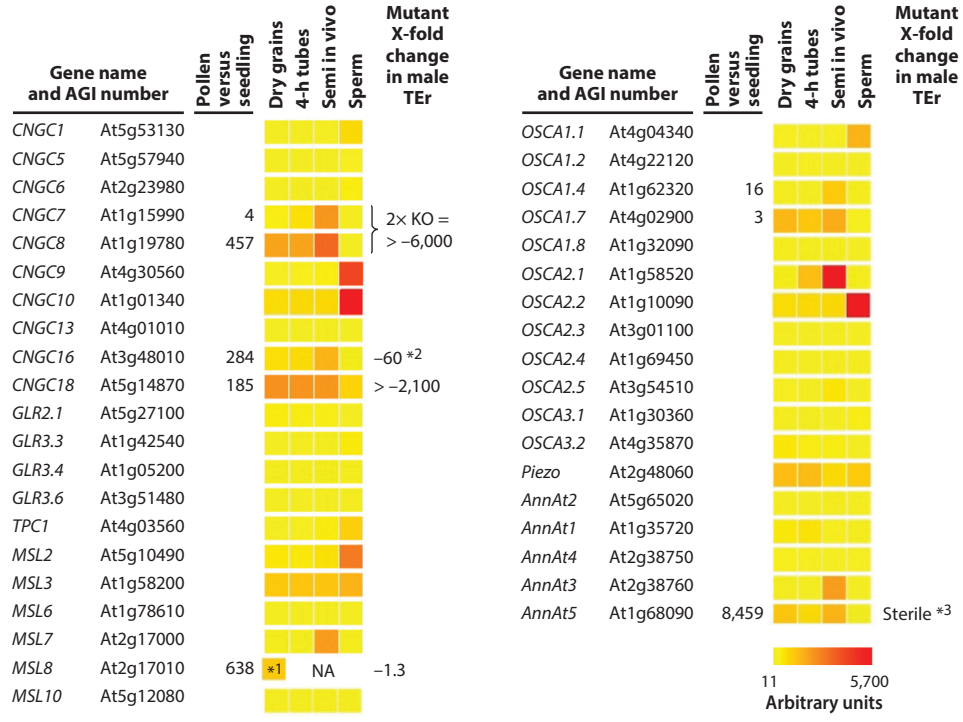

Figure 2

Relative mRNA expression levels for the most highly expressed Ca^{2+}-permeable channels in *Arabidopsis* pollen. The *Arabidopsis* genome encodes at least 77 candidate Ca^{2+}-permeable channels (19), including CNGCs, GLRs, TPCs, MSLs, OSCAs, a piezo-type channel, and annexins (see **Supplemental Table 2**). The 39 most highly expressed channels in pollen are listed, along with a heat map (**http://arabidopsis-heat-tree.org/**; based on Reference 115) showing their relative expression in pollen grains, pollen tubes, semi–in vivo growing tubes, and isolated sperm cells. (*1) indicates the heat map value for *MSL8* was estimated from RNA-seq data in References 57 and 84 because the gene was not included in the probe sets used for the heat map viewer, as denoted by NA. Pollen expression was corroborated for all 39 genes by RNA-seq (57, 84). Although some additional genes were detected by RNA-seq, they are not listed here because their expression levels were deemed too low for confident quantification (see **Supplemental Table 2**). The column titled "Pollen versus seedling" shows the X-fold increase in relative expression in pollen compared to seedlings for genes in which there is a preferential expression in pollen, based on RNA-seq values in Reference 84. For genes in which there is genetic evidence for a defect in pollen transmission, the fold-change in the TEr is shown, with a negative sign indicating a decrease in transmission of the mutant pollen relative to a control. The TEr is calculated as the number of transmission events in an outcross corresponding to mutant or transgenic pollen relative to the number of control or WT pollen. The TEr shown for *cngc7/8* is for pollen harboring a double gene knockout (2× KO). Note that the symbol (>) indicates that a transmission event has not yet been detected at the threshold value shown. (*2) indicates that the transmission defect was dependent on a stress condition. (*3) indicates a sterility phenotype based on RNAi was inferred without reporting a transmission efficiency. References for transmission defects correspond to *cngc7/8* (135), *cngc16* (136), *cngc18* (36, 37; J.F. Harper, unpublished data), *msl8* (47), and *annAt5* (150). Abbreviations: AnnAt, annexins from *Arabidopsis thaliana*; CNGC, cyclic nucleotide gated channel; GLR, glutamate like receptor; mRNA, messenger RNA; MSL, mechanosensitive-like channel [also known as small conductance (MscS)-like protein]; NA, not available; OSCA, reduced hyperosmolality-induced $[Ca^{2+}]_i$ increase channel; RNA-seq, RNA sequencing of transcriptome; RNAi, RNA interference; TEr, transmission efficiency ratio; TPC, two-pore channel; WT, wild type.

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2.1. Balancing Cellular Expansion with Cell Wall Flexibility

Ca^{2+} circuit: transport system that controls Ca^{2+} influx and efflux across a membrane

Rho GTPase of plants (ROPs): a subfamily of Rho-related small G-proteins that function as a molecular switch resulting from changes in conformation upon GTP binding and hydrolysis

Supplemental Material >

Localization of growth machinery to a single region in the tube's apex (i.e., growth cone) (50) is a fundamental feature that distinguishes pollen tube tip growth from growth of a typical plant cell. The growth cone becomes a construction zone where new plasma membrane and cell wall materials are delivered through the secretory pathway. This cargo delivery occurs primarily at the very tip (classical model) or to a lateral region immediately adjacent to the tip (alternative model) or to both regions (42) (**Figure 3**). Exocytosis of cargo vesicles is estimated to add a tenfold excess of new plasma membrane over what is needed for expansion. This excess addition of membrane is balanced by a compensating rate of removal by endocytosis. To efficiently recycle membranes for rapid tip growth, it has been proposed that the growing tip houses a specialized compartment of the *trans*-Golgi network that recycles membranes without trafficking endosomes to the vacuole (128).

A second fundamental feature of pollen tube tip growth is a spatial separation between new wall materials (for example, methylesterified pectins) deposition at the tip and their subsequent maturation through the de-esterification of pectins by pectin methylesterases (PMEs), such as VANGUARD1 (61), along the subapical and shank regions (10, 49, 99) (**Figure 3**; **Supplemental Table 1** provides a list of key genes and their identifiers). This de-esterification creates carboxyl groups that allow pectins to form Ca^{2+}-salt bridges with other polymers, creating a pectin gel that helps rigidify the wall. The activity of secreted PMEs at the tip is initially inhibited by interactions with PME inhibitors (PMEIs) (10, 49, 99). As PME/PMEI complexes dissociate, de-esterification activity increases and wall rigidification occurs in the subapical regions.

The construction of the pollen cell wall also includes many different polysaccharides and modifications (reviewed in 99), including acetylation (41), borate diester cross-links (26), secretion of fucosylated xyloglucans (21), and localized synthesis of cellulose and callose (11).

Secreted proteins [for example, in *Arabidopsis* pollen, leucine-rich repeat extensins (LRXs)] are potentially involved in modifying the structural properties of the pollen wall and plasma membrane and signaling systems. The combined mRNA expression levels for *LRX8*, *9*, *10*, *11* make *LXR*s one of the most abundant mRNAs in pollen (ranked first, thirteenth, and second in pollen mRNA abundance, respectively, in References 57, 84, 115). Combinations of *lrx* mutants display abnormal pollen tubes with an irregular deposition of callose and pectin (25, 121, 141). A quadruple *lrx* mutant showed a decrease in pollen transmission of approximately sixfold (calculated as TEr) (25). Manipulating Ca^{2+} availability in *lrx* mutant tubes partially suppresses growth defects, suggesting that LRX proteins influence Ca^{2+}-related processes (25). A complex of LRX and the peptide ligands rapid alkalinization factor 4 (RALF4) and RALF19 (92) activates two receptor-like kinases (RLKs) that are important for pollen tube growth, ANXUR1 (ANX1) and ANX2 (7, 92, 97). Future work may explore how RALF/LRX/RLK signaling functions in pollen, whether it regulates a Ca^{2+} circuit to transduce signals from the extracellular matrix across the plasma membrane to the cytosol (**Figure 3**), and how its function is integrated with other pollen tube–expressed proteins, such as the serine/threonine protein phosphatases ATUNIS1 and ATUNIS2 (35) and MARIS, a receptor-like cytoplasmic kinase (5) to maintain the pollen tube cell wall integrity.

Pollen cells secrete a variety of hydrolases, which likely degrade the cell wall of pollen or of the surrounding pistil cells and create elicitors or damage-associated molecular patterns (DAMPs) (23). An interesting question is how do pollen tubes respond to these signals to ensure compatible interactions with the pistil (i.e., how do they avoid being attacked as pathogens)?

2.2. Modulating Growth Cycles with Rho GTPases of Plants

A signaling network based on Rho GTPases of plants (ROPs) and their downstream effectors has emerged as a central regulatory mechanism underlying polarized cell expansion in plants (30, 31)

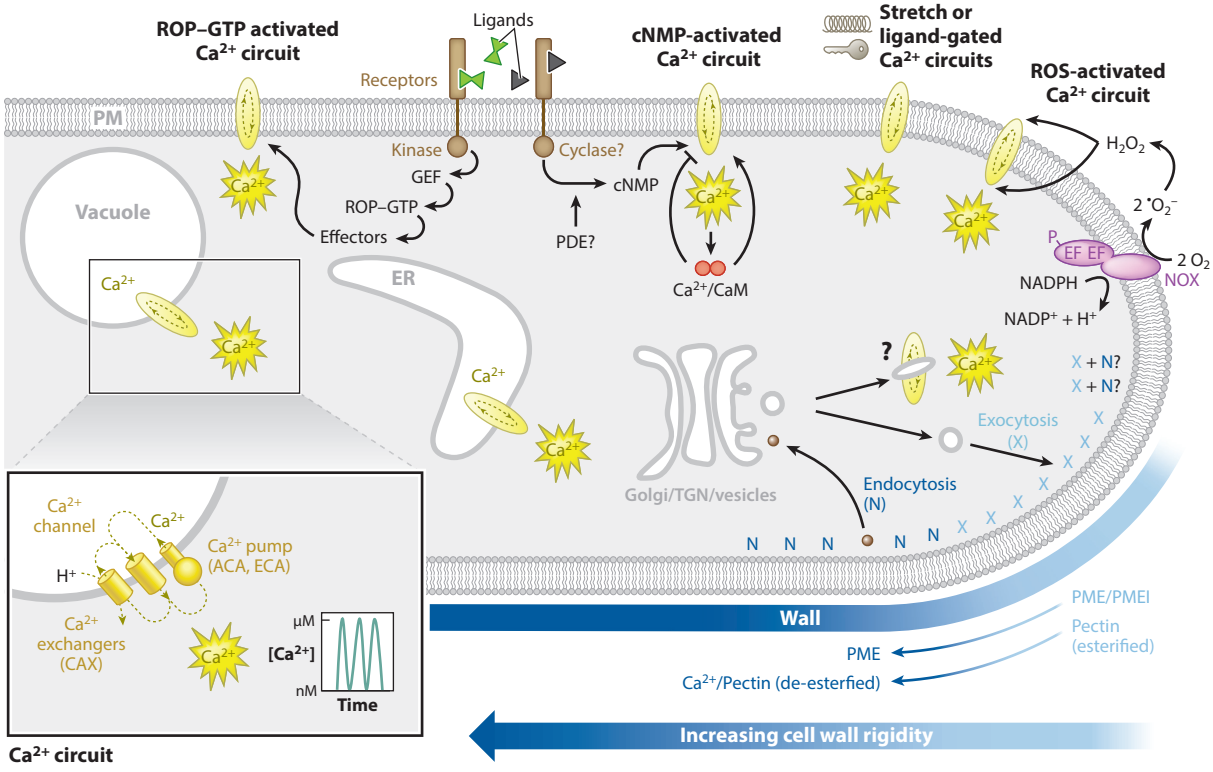

Figure 3

Ca^{2+} circuits of potential importance to regulating tip growth. Inset shows a Ca^{2+} circuit composed of channels for Ca^{2+} influx and pumps and exchangers for Ca^{2+} efflux (19). There are Ca^{2+} circuits located in multiple membranes, including the PM, vacuole, ER, and potentially other compartments associated with the secretory pathway, plastids, and mitochondria. Each Ca^{2+} circuit is subject to regulation by different factors that inhibit or activate influx and efflux pathways, such as Ca^{2+}/CaM, which activates ACA-type Ca^{2+} efflux pumps. The feedback from activating Ca^{2+}/CaM also inhibits Ca^{2+} influx through a cyclic nucleotide (cNMP) gated channel, such as in a cNMP activated Ca^{2+} circuit. While the source of cNMPs in pollen is not clear, there is evidence that some receptor kinases can have both kinase and cyclase activities (88). It is also not known which genes in plants might encode PDEs to terminate a cNMP signal. A NOX is shown as a source of ROS that can function as a signaling molecule or as a source of free radicals that can chemically modify the cell wall. Additional features corresponding to the EF-hand Ca^{2+}-binding site and a Ca^{2+}-dependent protein kinase–phosphorylation site (denoted as P) are important for Ca^{2+} activation of NOX activity (147). A portion of the cell wall is shown to illustrate one of the important structural features in which esterified pectins are secreted at the tip and then become de-esterified and more rigid as PMEs dissociate from PMEIs in subapical regions. Potential sites of exocytosis and endocytosis are marked. Abbreviations: ACA, autoinhibited Ca^{2+} ATPase; CaM, calmodulin; CAX, Ca^{2+} exchanger; cNMP, 3′,5′-cyclic nucleotide monophosphate; ECA, ER-type Ca^{2+} ATPase; EF, the EF-hand Ca^{2+}-binding site; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; N, endocytosis; NOX, nicotinamide adenine dinucleotide phosphate oxidase (also known as respiratory burst oxidase homolog, RBOH); PDE, phosphodiesterase; PM, plasma membrane; PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; ROP, Rho GTPase of plants; ROS, reactive oxygen species; TGN, *trans*-Golgi network; X, exocytosis.

(**Figure 3**). While there are many regulatory pathways critical to tip growth, ROPs are discussed here to illustrate how one of the best characterized molecular switches can be inhibited by Ca^{2+} signals.

There are 11 ROPs in *Arabidopsis*, and, among these, ROP1 has a dominant role in regulating pollen tube tip growth. ROPs adopt an active conformation when bound to GTP and become inactive when the GTP is hydrolyzed to GDP (**Figure 3**). The exchange of GDP with GTP is catalyzed by a guanine nucleotide exchange factor (GEF). The newly activated ROP–GTP

interacts with a variety of effectors, only a few of which are well established in plants (30). Importantly, ROP–effector interactions are time limited because the intrinsic GTPase activity of ROPs hydrolyzes GTP to GDP. The duration of the ROP–GTP active conformation can be shortened through interactions with GTPase-accelerating proteins (GAPs) that increase the rate of GTP hydrolysis. ROP–GDP can also interact with GDP-dissociation inhibitors (GDIs) to reduce a ROP's availability for reactivation by GEFs (31). As noted in **Figure 3**, the regulatory activities of GAPs, GEFs, and GDIs can all be altered by phosphorylation or phosphatase activities. Activation or inhibition of this regulatory module is thus linked to several phosphoregulatory pathways, including Ca^{2+}-dependent protein kinases (CPKs), mitogen-activated protein kinases (MAPKs), and RLKs (13, 30).

$[Ca^{2+}]_{cyt}$ oscillation: a transient increase in cytoplasmic calcium concentration that occurs in a repetitive pattern

There are many potential ROP effectors involved in regulating tip growth (**Figure 3**). Two important effectors include ROP-interactive CRIB motif–containing protein 4 (RIC4), which promotes F-actin assembly, and RIC3, which stimulates Ca^{2+} transients that can trigger F-actin disassembly (68). The antagonistic functions of RIC3 and RIC4 are proposed to create a regulatory module in which the two effectors could fine-tune actin dynamics during tip growth (68). It is not yet clear if RIC3 directly interacts with a Ca^{2+}-permeable channel or indirectly activates Ca^{2+} influx by triggering another signaling pathway.

One of the downstream ROP–GTP effectors is a plasma membrane NADPH oxidase (NOX), which generates reactive oxygen species (ROS) that can modify the cell wall and activate signaling pathways (30, 147). ROS is proposed to activate a yet-to-be-identified Ca^{2+}-permeable channel. Interestingly, NOX is also activated directly by Ca^{2+} and CPKs, providing a potential positive feedback mechanism in which a ROS-initiated Ca^{2+} signal could trigger another ROS signal (87) and, thereby, also trigger a repeating oscillation, e.g., of ROS to Ca^{2+} to ROS to Ca^{2+}.

While many regulatory pathways are expected to provide feedback regulation to activate or inhibit ROP and downstream effectors, **Figure 4** illustrates how three Ca^{2+}-dependent mechanisms could act synergistically to inhibit ROP–GTP-dependent pathways. A reactivation of ROP signaling would occur after cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) is restored to basal levels by pumps and ion exchangers (**Figure 3**). The multiple ways in which Ca^{2+} feedback can attenuate the activities of ROP–GTP suggests a potential synchronizing function for tip-focused Ca^{2+} oscillations.

2.3. Does the Tip-Focused Ca^{2+} Oscillator Function as a Synchronizer?

A tip-focused oscillation in $[Ca^{2+}]_{cyt}$ has inspired speculations on whether growth cycles start or stop in response to a Ca^{2+} signal. This enduring question has been reviewed many times along with current knowledge of the cellular pathways that might be inhibited or activated by Ca^{2+} oscillations (e.g., 94, 127).

A $[Ca^{2+}]_{cyt}$ oscillation requires the coordinated activation of one or more Ca^{2+} circuits (**Figure 3**), which include Ca^{2+} influx through a channel and efflux through a Ca^{2+} pump or exchanger (19). In *Arabidopsis* pollen, there are at least 39 genes encoding candidate Ca^{2+}-permeable ion channels (**Figure 2**; **Supplemental Table 2**), including *cyclic nucleotide gated channels* (*CNGC*s), *glutamate-like receptors* (*GLR*s), *mechanosensitive-like channels* (*MSL*s), *reduced hyperosmolality-induced $[Ca^{2+}]_i$ increase channels* (*OSCA*s), a piezo-type channel, and annexins. It is not clear which Ca^{2+}-permeable channels are directly involved in tip-focused $[Ca^{2+}]_{cyt}$ oscillations (127). While a Ca^{2+} conductance across the plasma membrane is thought to be essential, it is not known if there is an additional Ca^{2+}-induced Ca^{2+} release from endomembrane compartments present at the tip (**Figure 3**). There is genetic evidence from mutations associated with *CNGC18* (37) and several *GLR*s (93, 148) that deficiencies in these channels can alter but not eliminate $[Ca^{2+}]_{cyt}$ oscillations. The large number of Ca^{2+}-influx pathways underscores the expectation that the tip-focused Ca^{2+} oscillation is just one of many Ca^{2+} circuits that function in pollen.

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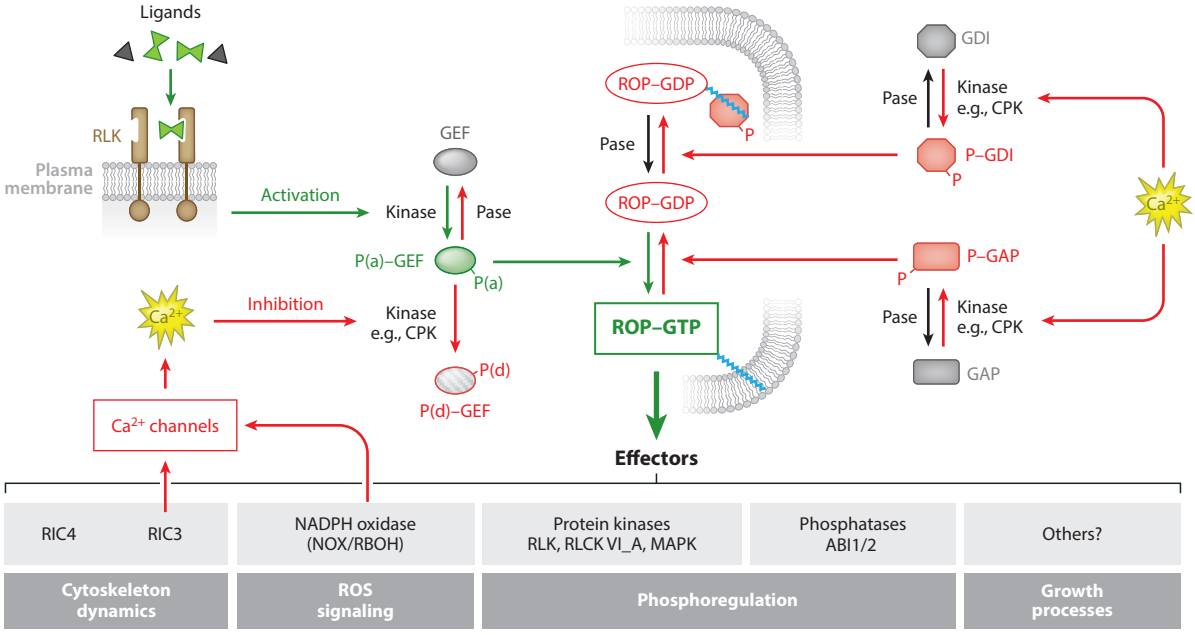


Figure 4

A ROP–effector signaling hub. Signaling pathways are shown that lead to activation (*green arrows*) and inhibition (*red arrows*) of ROP–GTP–effector-dependent cellular functions. Extracellular ligands can activate RLKs that phosphorylate a GEF (30). Phosphoactivation can be reversed by a Pase. Other kinases, such as CPKs, can phosphorylate other GEF sites to promote degradation (*hash marks*) (31). A phosphoactivated GEF promotes the exchange of GDP with GTP leading to an active ROP–GTP conformation that can interact with downstream effectors. A phosphoactivated GAP can inhibit the ROP by stimulating the ROP's intrinsic GTPase activity to hydrolyze GTP to GDP. A phosphoactivated GDI can stabilize the ROP–GDP in an inactive conformation. While there are likely many signaling systems that can modify this signaling hub, a Ca^{2+} transient (*star burst*) is shown to illustrate how Ca^{2+} signals at the pollen tip might inhibit the ROP pathway. There is evidence that ROP effectors can induce Ca^{2+} transients, thereby providing a feedback mechanism by which a ROP–GTP can trigger its own deactivation. Abbreviations: ABI, abscisic acid–insensitive phosphatases; CPK, Ca^{2+}-dependent protein kinase; GAP, GTPase-accelerating protein; GDI, GDP-dissociation inhibitor; GEF, guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; NOX, nicotinamide adenine dinucleotide phosphate oxidase; P(a), phosphoactivated; Pase, phosphatase; P(d), phosphorylated to promote degradation; RBOH, respiratory burst oxidase homologs; RIC, Rho GTPase of plants–interactive CRIB motif–containing protein; RLCK VI_A, receptor-like cytosolic kinase, class VI_A; RLK, receptor-like kinase; ROP, Rho GTPase of plants; ROS, reactive oxygen species.

Regardless of the Ca^{2+} source, there are numerous cellular functions that are potentially regulated by tip-focused $[Ca^{2+}]_{cyt}$ oscillations (127). Several of these are associated with cytoskeletal dynamics (48). In *Arabidopsis*, the downstream targets also include functions associated with at least 230 pollen-expressed genes that encode proteins known to bind Ca^{2+} or that are implicated in Ca^{2+} signaling (57). For example, there are multiple primary sensors, such as calmodulins or calcineurin B–like proteins (CBLs), 25 CPKs, and 13 CBL-interacting protein kinases. Because CPKs by themselves can potentially phosphoregulate hundreds of additional downstream targets (17), the goal of understanding how Ca^{2+} signals impact tip growth is an enormously complex challenge for systems biology.

The tip-focused $[Ca^{2+}]_{cyt}$ oscillations were previously speculated to have an essential role in every growth cycle (94). However, Ca^{2+}-imaging experiments have documented exceptions in which pollen tubes displayed multiple growth cycles without a detectable Ca^{2+} oscillation (e.g., 18, 58). There are also examples in which the tubes did not grow but still showed robust Ca^{2+}

Female gametophyte: a multicellular structure derived from a meiotic product containing two female gametes and two types of accessory cells

oscillations. These observations suggest that Ca^{2+} oscillations are not required for a growth cycle and favor an alternative model in which Ca^{2+} oscillations function intermittently as a reset switch to resynchronize or reboot cellular processes. The ROP–GTP signaling module discussed previously (**Figure 4**) provides one example of a potential reset switch in which a Ca^{2+} oscillation could simultaneously turn off multiple downstream effectors involved in growth processes and then reboot in a synchronized fashion once $[Ca^{2+}]_{cyt}$ was restored to basal levels. It is possible that multiple growth cycles can occur before another synchronizing reboot is needed to ensure coordination. As an analogy, marching bands use drummers to produce audible signals to synchronize the movement and music of the entire band. In the absence of a drum beat, the band might continue to perform and march for a considerable period of time, but eventually the group becomes unsynchronized and chaotic.

The following sections expand upon these fundamentals of tip growth, identify challenges in understanding how tip growth is modified to follow guidance cues to the ovules, and explore how tip growth stops and triggers a bursting event to deliver sperm cells that fuse with the egg and central cell.

3. SETTING THE STAGE: INTERACTIONS WITH THE STIGMA MARK THE BEGINNING OF A LONG, GUIDED TREK THROUGH THE FEMALE REPRODUCTIVE TRACT

Pollen tip growth becomes more complicated when it interacts with the stigma, which has to distinguish compatible pollen (self from nonself), help compatible pollen generate a tube, and place compatible pollen on the right path to the female gametophyte. Interaction between the pollen cell surface (which was deposited by the sporophytic anther) and the extracellular matrix of the sporophytic stigma mediates pollen adhesion and hydration. These initial interactions have evolved to promote diversity through sporophytic self-incompatibility-signaling systems, which selectively promote pollen germination only when the pollen and stigma carry dissimilar alleles and interact with each other (54). If the pollen grains are compatible, then after adhering to the stigma, they will hydrate, germinate a tube that develops via tip growth in the style, transmitting tract, septum, funiculus, and micropyle (**Figure 1**) to complete fertilization.

3.1. Challenges Encountered During Pollen Tube Emergence

Pollen hydration on the stigma is regulated by both pollen and stigma factors (9). Pollen hydration triggers a rapid influx of Ca^{2+} into the pollen grain (51) followed by cytoplasmic reorganization (51) and ROS accumulation within the grain (38). Clues to understanding how the tube emergence site is established in an otherwise isotropic hydrated pollen grain may come from investigating establishment of a cytoplasmic Ca^{2+} gradient (51, 60) or localization of either ROP–interactive partner 1 (RIP1) (76) or the exocyst subunit SEC3A (77) at the site of pollen tube emergence.

Another challenge faced by the rapidly hydrating pollen grain is to avoid lysing from the osmotic shock caused by water entry. It is known that *Escherichia coli* prevents cellular lysis upon hypoosmotic shock by releasing osmolytes from the cell using mechanosensitive channels. The pollen-expressed *Arabidopsis* mechanosensitive-like channel 8 (MSL8) protein may play a similar role and prevent pollen grain lysis after hydration (47). Additional turgor homeostasis mechanisms likely include regulation of aquaporins (113) and sphingolipid signaling (12).

3.2. Rapid Pollen Tube Germination Is Critical for Reproductive Success

In vivo pollen germination in *Arabidopsis* is rapid on dry stigmas [approximately 30 min (33) compared with >3.5 h in vitro (116)]. Lipids in the pollen coat of *Arabidopsis* are key to germination

(114). Additional factors from the pistil promote *Arabidopsis* pollen germination, including sulfinylated azadecalins (116) and brassinosteroids (138). Rapid and prolific pollen germination in vivo in *Arabidopsis* suggest that compatible pollen–stigma interactions have evolved to achieve maximal fertilization efficiency.

3.3. Proper Navigation of Pollen Tubes Is Critical from the Get-Go

To avoid wandering tubes, stigma factors direct pollen tubes toward the ovary. For example, chemocyanin, a small basic protein from lily stigmas, directs emerged pollen tubes toward the transmitting tract (67). In addition to sensing guidance cues, pollen tubes need to penetrate multiple physical barriers. Penetration is expected to involve many cellular functions, including the secretion of digestive enzymes, and signaling systems to ensure compatible interactions and cellular homeostasis. These functions are beginning to be identified through genetics. For example, expression of an *O*-FUCOSYLTRANSFERASE 1 (AtOFT1) in *Arabidopsis* pollen tubes is critical for pollen tube penetration through the stigma–style interface (123). In addition, a mutation of ceramidase results in pollen tubes with *turgor regulation defect 1* (*tod1*) and a decreased ability to penetrate through the stigma and style (12).

3.4. Future Directions

Understanding how pollen tubes penetrate into the stigma will require investigating gene regulatory networks and complex cellular responses. Mutations that disrupt this process can be used to identify genes associated with both normal and specialized cellular functions, and these analyses will be important for defining both compatible and incompatible pollen–stigma interactions.

4. SUSTAINING WHAT WAS STARTED: POLLEN TUBE GROWTH IN THE STYLE, TRANSMITTING TRACT, AND SEPTUM

The transmitting tract is lined with specialized extracellular matrix, a complex mixture of polysaccharides, glycoproteins, and glycolipids thought to support pollen tube growth (69). The septum runs vertically along the entire length of the ovary, and ovules arise from the junction of the septum and ovary walls (**Figure 1*a***; **Supplemental Figure 1**). The critical function of these tissues in the female reproductive tract is to ensure that pollen tubes reach the ovules. In some plants, these tissues are the site of gametophytic self-incompatibility—if alleles carried by the haploid pollen tube match those in the diploid pistil, pollen tube growth is arrested in the style, transmitting tract, or septum (144).

Supplemental Material >

4.1. What Sustains the Extension of the Fastest Plant Cell?

The fuel for rapid pollen tube growth remains poorly understood. Although the *TRANSMITTING TRACT–SPECIFIC* (*TTS*) glycoproteins *TTS1* and *TTS2* from *Nicotiana tabacum* and *NaTTS* from *Nicotiana alata* stimulate pollen tube growth in vitro (14), it is not clear if their homologs in *Arabidopsis* also stimulate pollen tube growth (146). GABA and D-serine, two uncommon amino acids, have a role in promoting pollen tube growth. Exogenous GABA influences pollen tube growth in vitro, stimulating pollen tube growth at lower concentrations and inhibiting tube elongation at higher concentrations (108). Pistil-generated D-serine facilitates pollen tube growth in the style, transmitting tract, and ovules (93) potentially by functioning

as a GLR agonist in the apical region of pollen tubes and regulating pollen tube Ca^{2+} dynamics. The function of pollen tube–expressed GLRs is dependent on their subcellular localization, which in turn is regulated by their cognate CORNICHON HOMOLOG (CNIH) chaperones (148). However, the specific cellular functions of D-serine-triggered Ca^{2+} signaling remain to be determined.

Pollen tube reception: process by which the receptive synergid cell induces pollen tube growth arrest and the discharge of sperm cells

LURE: a small, cysteine-rich peptide produced by the synergid cells that is capable of mediating micropylar guidance of pollen tubes

4.2. Pollen Tube Adhesion to Transmitting Tract Cells Is Critical for Pollen Tube Growth

Pollen tubes adhere tightly to the extracellular matrix of the transmitting tract. The animal extracellular matrix contains adhesins, which mediate cell–cell interactions by binding to receptors. Two stylar molecules necessary for pollen tube adhesion to lily styles were identified using an in vitro adhesion bioassay. The larger of the two is pectin, and the smaller molecule is a 9-kd lipid transfer protein, stigma/stylar cysteine-rich adhesin (SCA). Both molecules are required for adhesion of pollen tubes to the stylar matrix because neither molecule alone can mediate adhesion individually (100, 112). How this adhesion promotes pollen tube growth is not known.

4.3. Growth in the Stigma and Style Renders the Pollen Tube Responsive to Guidance Cues from the Ovule and the Female Gametophyte

Pollen tubes grown in vitro share fundamental characteristics with those grown in the pistil: The cell is highly polarized and extends only at the tip, and the nucleus and a pair of sperm cells migrate as a tip-localized unit. However, noticeable differences in pollen tube growth have also been documented. Pollen tube length and rate of pollen tube elongation in a pistil are much higher than in an in vitro growth medium (109). In addition, pollen tubes germinated in vitro do not find the ovule micropyle efficiently; however, there is a significant increase in ovule targeting if pollen tubes are first grown through the stigma and style (52, 110). These results indicate that pollen tube growth on stigma and style renders them competent to perceive downstream signals from ovules, well before they encounter the ovules. It is now beginning to be understood how the pistil capacitates the pollen tube. Growth through the style promotes the expression of thousands of transcripts not well expressed in pollen tubes grown in vitro (72, 115), including the receptors for ovule attractants (132, 140) and transcription factors critical for pollen tube reception (70). In addition, in *Torenia fournieri*, an ovule-derived factor called AMOR also confers competency to pollen tubes grown through a cut style to respond to ovule attractants (98). AMOR was identified as an arabinogalactan polysaccharide, the terminal 4-*O*-methyl-glucuronosyl residue of which is sufficient to confer competence to pollen tubes. It remains to be determined whether the competency conferred by stigma and style is distinct from that by ovules.

4.4. Pollen Tube Emergence from the Transmitting Tract into the Ovary

Within the transmitting tract, many aspects of pollen tube growth remain unexplored. First is the question of how pollen tubes ultimately exit the transmitting tract to grow on the septum. Limited pollination experiments in *Arabidopsis* support a targeted exit from the transmitting tract (15) as does the finding that pollen tubes respond to LURE peptides accumulated at the base of the funiculus (131). Localized degradation of the cuticle along the transmitting tract might also promote or enable pollen tube emergence at the location of cuticle degradation (69). Second, what agents are employed by the pollen tube to burrow its way out of the transmitting tract to the

septum? Pollen tube mutants that fail to emerge from the transmitting tract will help answer this question.

4.5. Future Directions

Despite their importance in setting the stage for a fruitful journey, early stages of pollen tube growth in the pistil still remain poorly characterized. Besides the ovule-derived AMOR, other pistil signals that capacitate the pollen tube remain to be identified. Transcriptome analyses have also led to the identification of genes induced in the pollen tubes only after growth in pistils, as well as genes expressed in the transmitting tract tissues. For instance, identification of the zinc-finger transcription factor gene *NO TRANSMITTING TRACT* (*NTT*), which is required for transmitting tract development (15), and of the basic helix–loop–helix (bHLH) transcription factor genes *SPATULA* (*SPT*) and *HECTATE* (*HCT*), which are necessary for septum development, will help us understand pollen tube growth in the transmitting tract (16).

5. FATAL ATTRACTION: SYNERGID-SECRETED LURE PEPTIDES REORIENT THE TIP-FOCUSED CA^{2+} OSCILLATOR TO GUIDE POLLEN TUBES INTO THE MICROPYLE

5.1. Micropylar Guidance Is a Powerful Model for Understanding How Extracellular Signals Elicit Intracellular Responses

Pollen tube navigation from the stigma to the female gametophyte (**Figure 1*a***; Sections 3 and 4) requires multiple signaling systems. However, none of these course corrections are as dramatic as when the pollen tube turns toward the micropyle (**Figure 1*b***). In recent years, we have learned that synergid-secreted, small, cysteine-rich LURE proteins are the attractants (107, 131) and that multiple pollen tube–expressed receptor complexes (132, 140) perceive and transduce these molecular cues to reorient pollen tube growth.

5.2. Synergids Secrete Cysteine-Rich Proteins that Attract Pollen Tubes into the Micropyle

LUREs were first discovered in *Torenia fournieri* (107), a reference species with external synergids that was used to define the cellular interactions required for pollen tube attraction (53). Interestingly, LUREs had been found to attract pollen tubes species-preferentially, and this feature was later used to identify a clade of five LUREs in *Arabidopsis* (**Figure 5*c***) that lacked orthologs in closely related *Arabidopsis lyrata* (131). LURE proteins are necessary for pollen tube attraction in vivo (131) and sufficient for pollen tube attraction in vitro (107, 131). Still, a complete loss of micropylar attraction by eliminating LUREs in synergids has not been reported, possibly due to residual LURE activity in the available mutants or functional overlap between LUREs and other synergid-secreted proteins. The discovery of a gene regulatory network based in the neighboring central cell controlling production of pollen tube attractants points to the complexity of micropylar guidance (75).

In maize, a group of small cysteine-rich proteins termed EGG APPARATUS 1 (EA1) are necessary (89) and sufficient (90) for micropylar guidance of pollen tubes. EA1 and LUREs suggest that small cysteine-rich proteins are the micropylar guidance cues of flowering plants but that attractants have diverged extensively. Interestingly, female gametophytes can be reprogrammed to attract pollen tubes of a different species (even as divergent as maize and *Arabidopsis*) by transforming

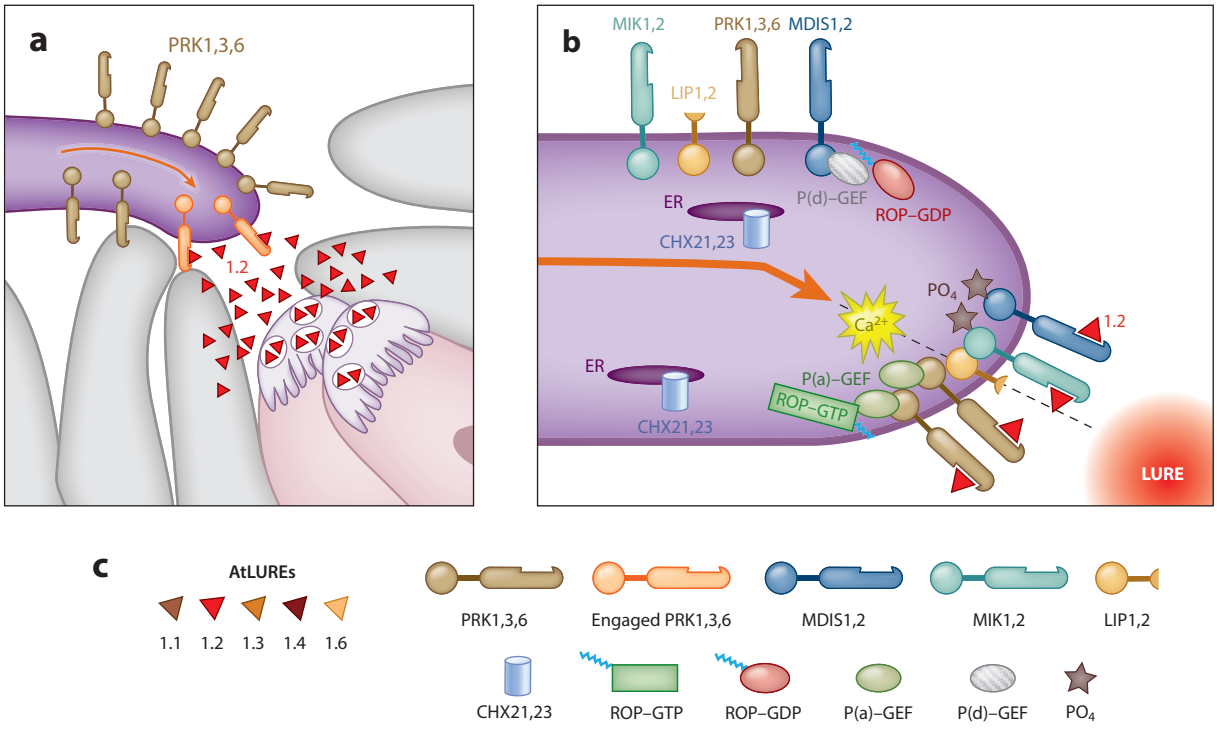


Figure 5

Synergid-secreted LURE proteins guide pollen tubes into the micropyle via receptor complexes that reorient Ca^{2+}, ROP activity, and the direction of extension. (*a*) A pollen tube with plasma membrane–localized LURE receptors is reorienting in response to a gradient of LURE peptides emanating from the filiform apparatus of the synergid cells. Engaged receptors are colored orange. LURE proteins are depicted on the surface of the integuments of the ovule and are predicted to diffuse through the extracellular matrix. (*b*) A model for reorientation of the direction of pollen tube extension (*orange arrow*) in response to LURE perception. Internal Ca^{2+} and external LURE gradients are aligned (*dashed line*) and thus define the direction of tip extension. This alignment is maintained by transmembrane receptor complexes that are maximally engaged by ligands proximal to the LURE gradient (*red triangles*). Receptors localized distally to the LURE gradient are unengaged and associated with inactive ROP (ROP–GDP). Engaged receptors autophosphorylate (140) and/or activate ROP–GEF [P(a)–GEF] and, thus, ROP (132), which is predicted to relocalize the tip-focused Ca^{2+} maximum. PRK6 has been shown to relocalize to the surface of the pollen tube tip, proximal to the LURE maximum closest to the LURE source (132). (*c*) Key molecules in the micropylar pollen tube–guidance mechanism. Abbreviations: CHX, cation-proton exchanger; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; LIP, LOST IN POLLEN TUBE GUIDANCE; MDIS, male discoverer; MIK, male discoverer–interacting receptor-like kinase; P(a), phosphoactivated; P(d), phosphorylated to promote degradation; PRK, pollen receptor kinase; ROP, Rho GTPase of plants.

Interspecific hybridization: successful interaction between male and female gametophytes of two different species that results in the fusion of their gametes

the female species with a single gene encoding an attractant (90, 131). Species-specificity of pollen tube attraction ensures that pollen tubes of the correct species will outcompete closely related pollen tubes for access to ovules, thus preventing interspecific hybridization. Such molecules are expected to diversify rapidly (130) and reinforce boundaries between species.

5.3. LUREs Are Perceived by Multiple, Closely Related Receptor-Like Kinase Complexes that Link Tube Extension with Reorientation

Perception of LUREs is mediated by a complex system involving multiple families of RLKs that interact with LURE ligands via extracellular leucine-rich repeats (132, 140, 149).

pollen receptor kinase 6 (*prk6*) mutant pollen tubes failed to reorient toward purified LURE1.2 (**Figure 5*b***) in a semi–in vivo attraction assay (132), implicating the eight-member *Arabidopsis* PRK family in micropylar guidance. Interestingly, the orthologous tomato family was already known to regulate pollen tube extension via autocrine and female-expressed ligands (44, 133). In *Arabidopsis*, loss of *prk6* but not of other *PRK*s resulted in failure to turn toward LURE1.2 in vitro; however, *prk6*,*prk3* double mutants exacerbated altered pollen tube growth near the micropyle (132). Interestingly, *prk6*,*prk3*,*prk1* triple mutant pollen tubes grew slowly through pistil tissue. These data point to a mechanistic link between the maintenance of pollen tube growth and LURE-induced change in the pollen tube growth trajectory. PRK6 interacts with ROP–GEF at the pollen tube tip (**Figure 5*b***), suggesting that activation of PRKs by LUREs results in localized activation of ROP and downstream Ca^{2+} signals (**Figure 5**) that reorient growth (86).

Polytubey: the state where more than one pollen tube enters an ovule

A parallel approach focused on protein phosphorylation by overexpressing kinase-defective mutant versions of candidate receptors in pollen tubes and yielded MALE DISCOVERER 1 (MDIS1) and MDIS2, a pair of closely related leucine-rich repeat receptor kinases (140). Protein interaction screening identified MDIS1-INTERACTING RLK 1 (MIK1) and MIK2, another closely related pair of RLKs in the same clade of the RLK family as MDIS1 and MDIS2 and closely related to the PRK clade (122, 140). Immunoprecipitation assays showed that MDIS1 and MDIS2 and MIK1 and MIK2 each interact with LURE1.2 and that ligand binding causes homodimerization and heterodimerization of receptors. Intriguingly, MDIS1 and MIK1 phosphorylation was found to be LURE1.2-dependent and dimer-dependent in vitro, suggesting that LUREs induce dimerization and activation of receptor signaling activity. Loss-of-function phenotypes for combinations of *MDS1* and *MDS2* and *MIK1* and *MIK2* showed subtle pollen tube–guidance defects but no pollen tube–extension defects like those observed in *prk* mutant tubes.

5.4. Polytubey Is Prevented by Tight Regulation of Synergid Viability

Arabidopsis ovules typically attract a single pollen tube, suggesting the existence of a mechanism that prevents arrival of multiple pollen tubes. However, if a pollen tube releases sperm that fail to fertilize female gametes, the ovule will attract a second pollen tube, suggesting that successful fertilization initiates the block to polytubey (2, 63). One of the two synergids degenerates immediately upon pollen tube rupture (see Section 6); however, the lifespan of the remaining synergid (the persistent synergid cell) is dependent upon whether fertilization occurs. Fertilization initiates ethylene-dependent signaling that results in persistent synergid cell nuclear degeneration (139), which is followed rapidly by the central cell's engulfment of the persistent synergid cell (91). This two-phase mechanism eliminates the LURE source, thereby preventing attraction of additional pollen tubes after fertilization.

5.5. Future Directions

X-ray crystallography has defined a LURE1.2-binding surface on PRK6 (149). Future work will determine how LURE1.2 also interacts with related RLKs and how binding initiates diverse dimer combinations resulting in phosphorylation and activation of signaling cascades (**Figure 5*b***). It will be important to understand whether individual complexes have distinct signaling outcomes that modulate pollen tube extension versus turning and whether potential coreceptors [e.g., LIP1 and LIP2 (80)] (**Figure 5**) change signaling outcomes. Additionally, it will be interesting to probe how LURE receptors modulate ion fluxes that are critical for polarized growth (see Section 2), particularly in light of the observation that a double knockout of *chx21*,*chx23* (potassium transporters) completely blocks micropylar guidance without affecting growth (85, 131).

6. BEGINNING OF THE END OF THE JOURNEY: COMPLEX INTERACTIONS BETWEEN THE POLLEN TUBE AND THE SYNERGID CELL CULMINATE IN SPERM RELEASE

Once in the ovule, pollen tubes migrate slowly to the filiform apparatus, a highly invaginated membrane-rich region at the micropylar end of the synergid cells (64) (**Figure 1*b***), and cease growth at one of the two synergid cells (the receptive synergid cell) (110). The filiform apparatus is the site for accumulation of signaling peptides (e.g., cysteine-rich peptides) (3) and small molecules (e.g., ROS) (22) that mediate pollen tube reception in the receptive synergid cell (82). Pollen tube reception is another important prezygotic barrier to interspecific hybridization because pollen tubes fail to cease growth and release sperm in interspecific crosses (24, 142).

Changes in $[Ca^{2+}]_{cyt}$ in the receptive synergid cell and pollen tube mark the initiation of pollen tube reception (20, 45, 59, 105). A prolonged slow growth of the pollen tube in Phase I coincides with a steady increase in pollen tube $[Ca^{2+}]_{cyt}$ and commencement of $[Ca^{2+}]_{cyt}$ oscillations in both synergids (20, 105). This is followed by a short Phase II, during which there is an increase in pollen tube growth and in the amount of transient $[Ca^{2+}]_{cyt}$ in the pollen tube and receptive synergid cell (105). Phase III comprises a final spike in pollen tube $[Ca^{2+}]_{cyt}$ and a decrease in receptive synergid $[Ca^{2+}]_{cyt}$ (105).

The receptive synergid cell degenerates either when the pollen tube enters the synergid cell or soon thereafter (27), and it was shown that successful pollen tube discharge accompanies or follows synergid degeneration (71). The temporal relationship between receptive synergid cell degeneration and receptive synergid cell $[Ca^{2+}]_{cyt}$ changes is not clear; most likely, synergid cell degeneration occurs at or soon after the final decrease in $[Ca^{2+}]_{cyt}$ in the receptive synergid cell (105).

6.1. FERONIA-LORELEI-Dependent Signaling Is Required for Pollen Tube Growth Arrest in the Receptive Synergid Cell

Only a handful of female gametophyte–expressed genes are known to regulate pollen tube reception (**Figure 6*a***). Loss of function of the *FERONIA* (*FER*) RLK causes synergids to fail to induce pollen tube reception, and, consequently, seeds are not produced (24, 56). Many mutants cause similar pollen tube reception phenotypes due to defects in the synergids: These include *scylla* (*syl*), *lorelei* (*lre*), *nortia* (*nta*), *evan* (*evn*), *turan* (*tun*), and *early nodulin-like proteins* (*ens*) (8, 55, 65, 79, 117, 134). *NTA* encodes a transmembrane member of the Mildew Resistance Locus O family (65). *EVN* and *TUN* encode the UDP-glycosyltransferase superfamily protein and a dolichol kinase, respectively, which might N-glycosylate synergid-expressed proteins functioning in pollen tube reception (79). *LRE* and *EN*s encode glycosylphosphatidylinositol (GPI)-anchored membrane proteins (55, 81).

Cooperative action of FER and LRE is critical for pollen tube reception (**Figure 6**). In mature, unpollinated ovules before pollen tube arrival LRE functions as a FER chaperone by enabling its movement from the endoplasmic reticulum to the filiform apparatus of the synergids (73). Once in the filiform apparatus, FER and LRE are part of the ROP-signaling complex that regulates ROS production in the filiform apparatus of synergids, which is important for pollen tube reception (22). Consistent with these functions, the extracellular domain of FER interacts with that of LRE (73).

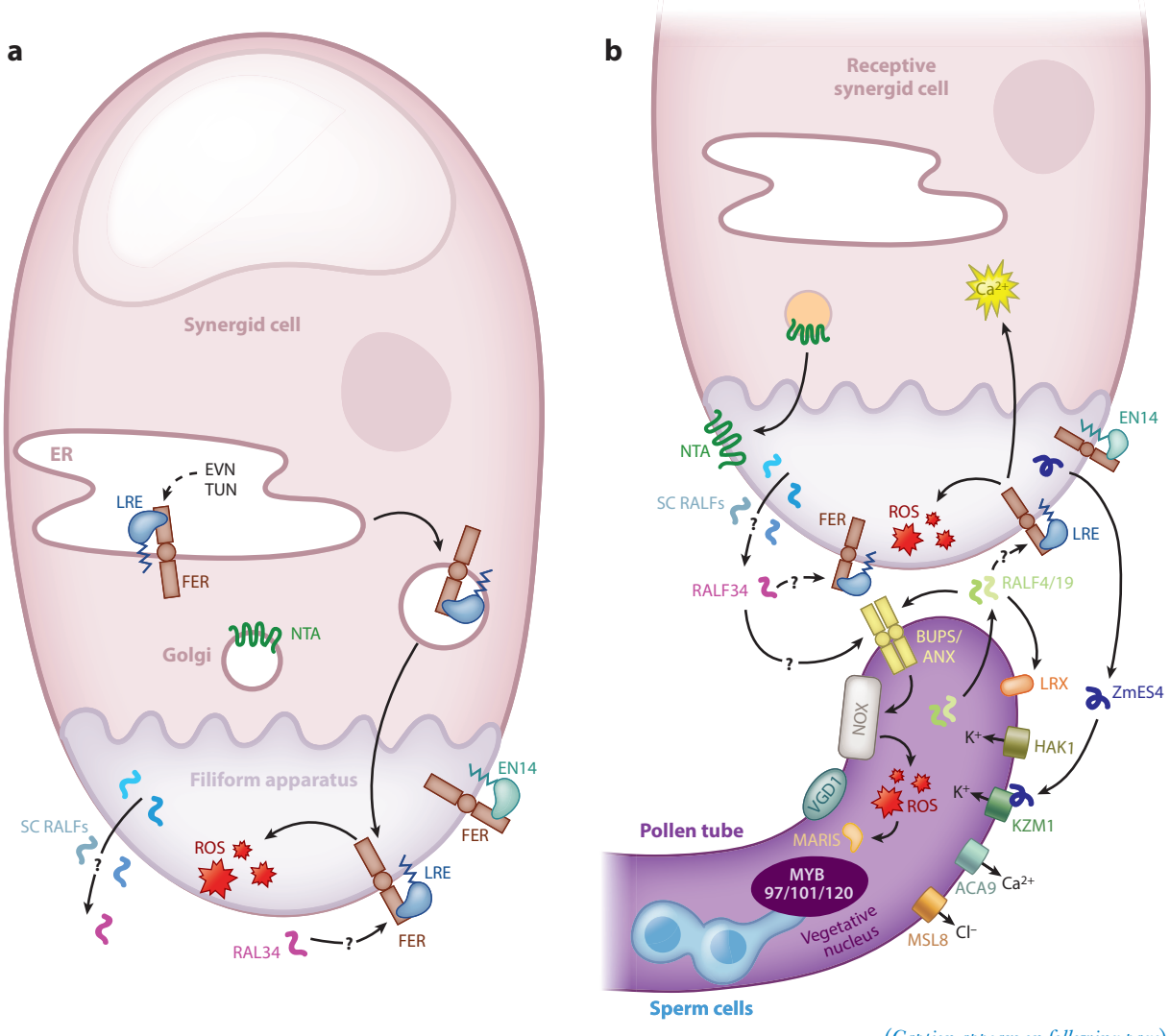
When the pollen tube interacts with the receptive synergid cell, changes in the synergid $[Ca^{2+}]_{cyt}$ and initiation of Ca^{2+} oscillations are dependent on FER and LRE (105). Additionally, FER is necessary for the preferential relocalization of NTA to the filiform apparatus upon pollen tube arrival, indicating that NTA functions downstream of FER (65) (**Figure 6*b***). Recently, it has been shown that EN14 localizes to the filiform apparatus of synergids, where it interacts

with FER and functions in pollen tube reception (55). Importantly, pollen tube–expressed *LRE* complemented the pollen tube reception defects of *lre* ovules (82), pointing to LRE's critical role in signaling after pollen tube arrival, distinct from its intracellular role as a chaperone.

Male gametophyte: the haploid pollen cell and its cargo of two sperm cells; the three cells are derived from a single meiotic product (microspore)

6.2. The Pollen Tube Components of FER–LRE Pollen Tube Reception Signaling Remain To Be Discovered

Interestingly, *abstinence by mutual consent* (*amc*) mutants showed pollen tube reception defects only when an *amc* pollen tube encountered an *amc* female gametophyte, indicating that pollen tube reception requires interactions between the male gametophyte and the female gametophyte and that both gametophytes share common signaling components (6). Further insight into the male gametophyte's role in pollen tube reception came from analysis of three closely related R2R3-MYB-type transcription factors (*MYB97*, *MYB101*, and *MYB120*) that are induced in pollen tubes



(*Caption appears on following page*)

Figure 6 (*Figure appears on preceding page*)

Pollen tube–synergid interactions mediate sperm release. (*a*) Before pollen tube arrival in the synergid cell, LORELEI (LRE) functions as a chaperone for FERONIA (FER) and promotes secretion from the endoplasmic reticulum (ER) to the filiform apparatus (*wavy lines*). ER-localized EVAN (EVN) and TURAN (TUN) are proposed to N-glycosylate proteins involved in sperm release. In the synergid plasma membrane, LRE and FER are coreceptors of a signaling pathway that produces reactive oxygen species (ROS) in the filiform apparatus. FER is also bound by another glycosylphosphatidylinositol (GPI)-anchored membrane protein, EARLY NODULIN-LIKE 14 (EN14), in the filiform apparatus of the synergid cell (SC). The ligands of the FER signaling pathway are not known. It is speculated that they could be synergid-derived rapid alkalinization factors (RALFs) or ovule-derived RALF34 (*question marks*). NORTIA (NTA) is a membrane protein that is primarily localized in the Golgi of synergid cytoplasm prior to pollen tube arrival. (*b*) After pollen tube arrival in the receptive SC, NTA is relocalized to the filiform apparatus. FER–LRE signaling is induced by the arrival of the pollen tube. However, unknown ligands (*question marks*) derived from the synergid cell and/or pollen tube mediate this induction. Unknown pollen tube components under the control of MYB97, MYB101, and MYB120 transcription factors also mediate reception of the pollen tube by the synergid cell. The integrity of the pollen tube cell wall—up until reaching the synergid cell—is mediated by RALF14/RALF19/ANXUR (ANX)/BUDDHA'S PAPER SEAL (BUPS) complex, aquaporins, leucine-rich repeat extension (LRXs), Vanguard 1 (VGD1), and mechanosensitive-like channel 8 (MSL8). However, upon arrival in the synergid cell, it is proposed that the RALF4 and RALF19 ligands are displaced by RALF34 to induce pollen tube lysis. The ANX-based signaling pathway coupled with nicotinamide adenine dinucleotide phosphate oxidase (NOX) is important for ROS production, which also may be involved in pollen tube lysis. *Zea mays* embryo sac (ZmES4) is another synergid-derived small peptide signal that interacts with *Zea mays* potassium transporter 1 (KZM1) to induce pollen tube lysis. Other proteins that may be involved in pollen tube lysis include high-affinity potassium transporter 1 (HAK1), autoinhibited calcium ATPase (ACA9), and MSL8.

grown through the stigma/style (115). No effect on seed set was seen in single and double *myb* mutants. However, in the triple *myb* mutant, in approximately 70% of ovules, the pollen tube coiled in the female gametophyte and failed to discharge sperm cells (70, 78). The genes regulated by this group of MYBs have begun to be analyzed (72); however, connections to FER–LRE signaling have yet to be made.

6.3. Cell Wall Integrity Is Abruptly Lost During Pollen Tube Reception

After reception by the receptive synergid cell, the pollen tube ruptures and releases the sperm cells (46, 110, 118). A synergid-expressed defensin-like protein, ZmES4, interacts with the pollen tube–expressed potassium Shaker channel KZM1 and induces the pollen tube to rupture (1) (**Figure 6**). Consistent with its role in pollen tube discharge, recombinant ZmES4-triggered pollen tube rupture in vitro and loss of ZmES4 resulted in a reduced seed set (1). In rice, the pollen tube tip-localized *Ruptured Pollen tube* (*RUPO*) RLK interacts with the potassium channel HAK1 to control potassium homeostasis and pollen tube cell wall integrity (81).

Five other pollen tube–expressed genes in *Arabidopsis* function in pollen tube discharge. Loss of *AUTOINHIBITED* Ca^{2+} *ATPASE 9* (*ACA9*), which encodes a Ca^{2+} efflux pump, causes pollen tube discharge defects in some *aca9* pollen tubes (120). While both triple *myb* and *aca9* tubes often fail to burst, they differ in that *aca9* mutants show a normal growth arrest (120) but the triple *myb* pollen keeps on growing (70, 78). Two pairs of *Arabidopsis* pollen tube–expressed CrRLK1L protein kinases, ANX1 and ANX2 and BUDDHA'S PAPER SEAL 1 (BUPS1) and BUPS2, are important for maintaining the integrity of the pollen tube during growth (39, 151). Pollen tube integrity is maintained if the autocrine signaling complex is comprised of BUPS1 and BUPS2 and ANX1 and ANX2 bound to the pollen tube–expressed ligands RALF4 and RALF19 (39) (**Figure 6*b***). Unlike RALF4 and RALF19, ovule-expressed RALF34 induces pollen tube rupture and binds BUPS1, BUPS2, ANX1, and ANX2 receptors with a greater affinity. This finding led to the model that displacement of RALF4 and RALF19 by RALF34 causes pollen tube discharge in the receptive synergid cell (**Figure 6*b***). However, this model has yet to be tested via a loss-of-function analysis of *RALF34* to determine whether it is necessary for pollen tube reception.

6.4. Receptive Synergid Degeneration Is Necessary for Successful Double Fertilization

In *Arabidopsis*, receptive synergid cell degeneration occurs after the pollen tube arrives at the female gametophyte (110). A role for the female gametophyte in synergid degeneration was confirmed when it was found that pollen tube arrival in the receptive synergid cell promotes synergid degeneration (71). A role for the male gametophyte in synergid degeneration was shown by analyzing wild-type ovules targeted by *myb* triple mutant pollen tubes (70). Two intact synergids remained in 42% of wild-type ovules containing undischarged *myb* triple mutant pollen tubes, indicating that this subset of *myb* triple mutant pollen tubes is deficient in promoting synergid degeneration (70).

6.5. Future Directions

The downstream components of the FER–LRE signaling pathway are yet to be identified (**Figure 6**). The identity and source (pollen tube and/or synergids) of ligands for the FER–LRE and NTA signaling pathways are not known (**Figure 6**). Similarly, the synergid-produced ligands that trigger pollen tube discharge in *Arabidopsis* (the functional equivalent of synergid-derived ZmES4) remain to be identified. Characterization of these signaling pathways will also unravel the molecular basis of pollen tube reception barriers in interspecific crosses.

7. THE END OF THE AFFAIR: GAMETE ATTACHMENT AND FUSION MECHANISMS

7.1. There Are Seven Critical Minutes Between Sperm Release and Gamete Fusion

Interactions between sperm and female gametes (the egg and central cell) (**Figures 1*a*** and **7*a***) were revealed by live imaging of sperm cells with bright, genetically labeled nuclei (46) (**Figures 1*b*** and **7*a***). The released sperm were tracked as they moved to the site of gamete fusion. They halted proximal to both the egg and the central cell for approximately seven minutes before they fused with female gametes, and then the nuclei resumed their migration (46).

7.2. Gamete Attachment Precedes Gamete Fusion

Gamete attachment was first appreciated in flowering plants following analysis of *Arabidopsis GAMETE EXPRESSED 2* (*GEX2*) (101) (**Figure 7*a***). GEX2 is a sperm-specific transmembrane protein that shares filamin-repeat domains with FUS1, a *Chlamydomonas reinhardtii* (*Chlamydomonas*) protein essential for gamete attachment (95). *gex2* sperm are delivered to ovules; however, they were found to be defective in attaching to the egg and central cell, and the mutant allele was shown to be transmitted less frequently than the wild-type allele by a difference of approximately 1.9-fold—a mild, but significant transmission defect. *gex2* sperm sometimes initiated the fertilization of one female gamete but not the other, resulting in aborted seeds. It will be interesting to determine whether GEX2 is conserved among flowering plants or whether, as in algae, gamete attachment is under evolutionary pressure to diversify in order to maintain species boundaries (32).

7.3. Gamete Fusion Is Mediated by HAP2–GCS1, an Ancient Sperm-Specific Protein Homologous to Viral Proteins that Mediate Fusion with Host Cells

hapless 2 (*hap2*) mutant alleles are not transmitted to progeny through the male parent (TEr = 0), yet do not affect female gametophyte function (62). The role of HAP2 was clarified when it

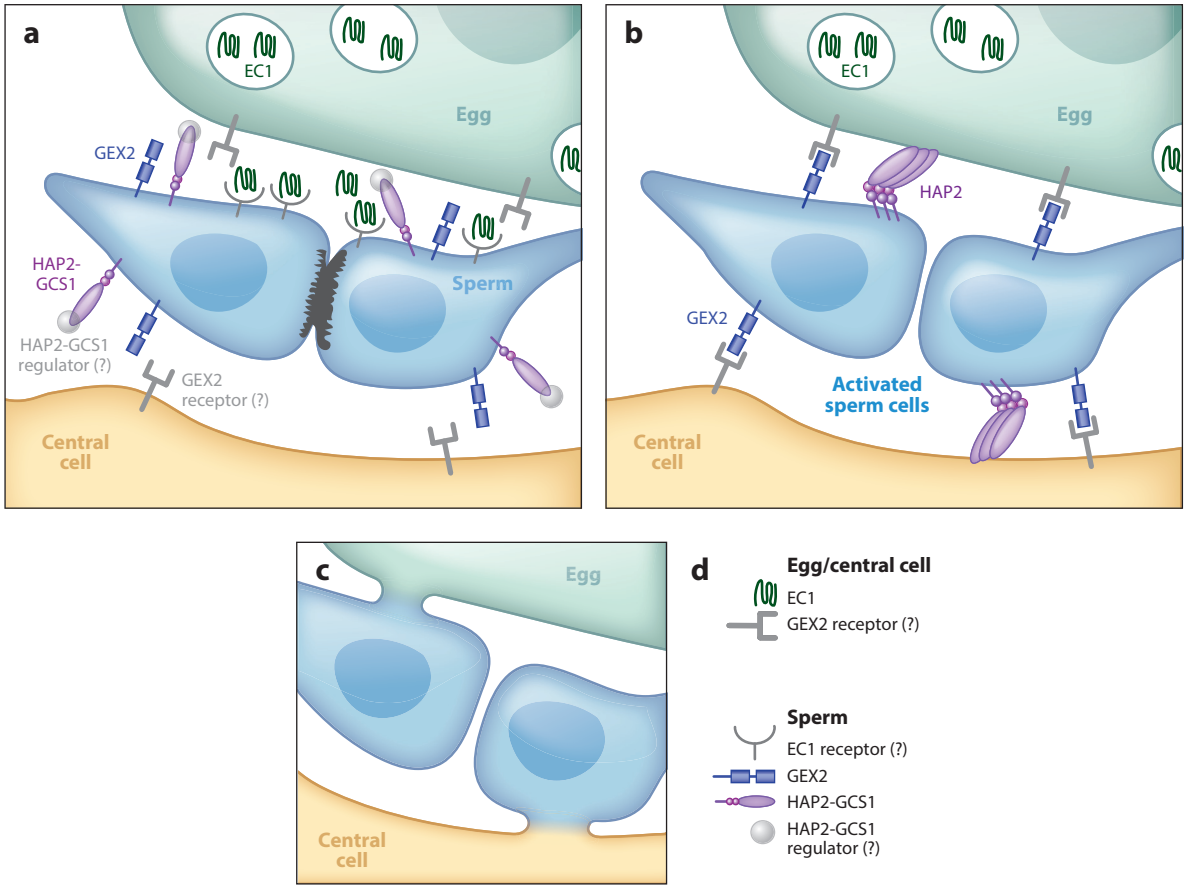


Figure 7

Gamete–gamete interactions leading to fusion of gamete PM. (*a*) The sperm have been deposited to the site of gamete interactions following rupture of the pollen tube and collapse of the receptive synergid, which opens a space between the egg and central cell. The gametes have little or no cell wall at this site where they interact with each other. Pollen tube rupture triggers secretion of EC1 from the egg cell. GEX2 and HAP2 are depicted as integral sperm PM proteins. The sperm cells are attached to each other by a membrane-rich attachment zone. GEX2 has yet to interact with its predicted egg-expressed and central cell–expressed receptor; however, EC1 is predicted to interact immediately with an as yet unidentified sperm-expressed receptor, leading to sperm activation. (*b*) Activated sperm are proposed to detach from one another as they attach to female gametes via GEX2. This may require remodeling of the sperm PM attachment region, as depicted. HAP2 is released from a putative negative regulator as it homotrimerizes and inserts into either the egg or the central cell PM. (*c*) PM fusion initiates with a fusion pore and is complete when the membrane of one sperm is incorporated into that of the egg while the membrane of the other sperm is incorporated into the central cell. Fertilization is complete when sperm nuclei migrate to female gamete nuclei within the zygote or primary endosperm cytoplasm. (*d*) Known and proposed (*gray*) molecular components of gamete interaction mechanisms. Abbreviations: EC1, EGG CELL 1; GCS1, GENERATIVE CELL–SPECIFIC 1; GEX2, GAMETE EXPRESSED 2; HAP2, HAPLESS 2; PM, plasma membrane.

was found that sperm containing *generative cell–specific 1* (*gcs1*), an independently identified mutant allele of *hap2*, failed to fertilize either the egg or the central cell when delivered to ovules (102) (**Figures 1*a*** and **7*a***). However, HAP2's specific role as a gamete fusion protein was first appreciated when *Chlamydomonas hap2* mutant gametes underwent all aspects of gamete interactions except for plasma membrane fusion; the membranes remained approximately 10 nm apart, which is the size of the gap that must be overcome by an active membrane fusion mechanism (83). *hap2*

orthologs have been identified in genomes representative of all eukaryotic clades except fungi (28, 83); so, HAP2 likely served as a gamete fusion protein in the earliest sexual eukaryotes.

The biochemical function of HAP2 was recently discovered (29) when *Chlamydomonas* HAP2 was shown to share structural similarities with class II viral fusion proteins, which have three extracellular domains; a single transmembrane domain; and a smaller, variable intracellular domain (66) (**Figure 7*a*,*b***). Upon entry into the host endosome, class II viral fusion proteins homotrimerize as a hydrophobic patch of residues at the apex of each monomer inserts into the host membrane. A conformational change of the trimer then drives fusion of the host membrane and the viral envelope (66). The structure of *Arabidopsis* HAP2 was also recently solved, and it was shown that an apical amphipathic helix is required for insertion into membranes in vitro and for fusion with the egg and central cell in vivo (28).

These findings suggest a model for double fertilization in which *Arabidopsis* HAP2 mediates the fusion of one sperm cell with the egg plasma membrane and the other sperm cell with the central cell by a unilateral mechanism that does not require a binding protein on the surface of the female gametes (**Figure 7*b*,*c***). This model raises a number of interesting questions. For example, if HAP2 is a unilateral fusion protein, how is its activity regulated so that the sperm cells do not fuse with each other while they are in the pollen tube? What guides the two distinct fusion events of the sperm and female gametes?

7.4. Egg Cell-Expressed EC1 Proteins Are Essential for Sperm Activation

After release from a pollen tube, sperm need to be activated to fuse with female gametes. During sperm development, the smaller generative cell is engulfed by the larger pollen cell (137); thus, sperm develop within a secondary membrane of pollen origin (124) (**Figure 1*a***). This membrane must be removed before the sperm plasma membrane can fuse with the female gamete. Second, the two sperm cells remain attached by a membrane-rich region that accumulates sperm membrane proteins, including HAP2 (4, 28, 125), and this region must be severed before sperm can individually attach and fuse with female gametes (compare **Figure 7*a*,*b***). Finally, HAP2 fusion activity must be activated.

Egg-secreted EC1 cysteine-rich proteins (EC1.1–1.5) are required for one or more aspects of sperm activation (125). EC1 proteins are released from the egg upon sperm release, and ovules with reduced *EC1* function were shown to be severely defective in fusion with wild-type sperm. It is likely that a sperm-expressed receptor interacts with EC1 (**Figure 7*a***) to initiate a signal transduction cascade that activates sperm. It will be important to determine whether EC1 plays a direct role in sperm pair severing, sperm attachment, and/or activation of HAP2 fusion activity.

7.5. Future Directions: The Block to Polyspermy in Flowering Plants

In cases when rare polytubey events have been analyzed, the rates of polyspermy (fusion of multiple sperm with one female gamete) are extremely low (43, 104), suggesting an active mechanism to block polyspermy. Polyspermy blocks in animals involve rapid membrane depolarization followed by slower but more permanent walling off of the zygote (145). Gamete fusion is accompanied by concomitant spikes in $[Ca^{2+}]_{cyt}$ in both the sperm and the egg (20, 45). It will be interesting to determine whether this is the cause or the consequence of membrane depolarization and if it results in immediate cessation of fusibility. Such a rapid block to membrane fusion may explain how both sperm do not fuse with the egg or central cell: After the first fusion event, the remaining sperm would fuse with the unfused female gamete.

8. CLOSING PERSPECTIVE: IMPORTANCE OF UNDERSTANDING REPRODUCTIVE STRESS TOLERANCE IN A CHANGING CLIMATE

Fertilization in many crop plants is highly sensitive to weather extremes (40, 103, 152). Even a single hot day or cold night can disrupt reproductive success. In many cases, pollen cells are considered some of the most vulnerable plant cells to short-term temperature stress (103, 152), with each of the signaling challenges highlighted in this review representing a potential point of temperature sensitivity. For example, it is possible that stress-induced Ca^{2+} transients (96) might interfere with other Ca^{2+} signals in pollen, such as those signals involved in regulating ROP–GTP–effector pathways (**Figures 4** and ***5b***).

While temperature stress perception might be similar in pollen and vegetative cells (34, 96, 103), a temperature stress response in pollen appears to be significantly different (57). In *Arabidopsis*, transcriptomes of heat-stressed pollen and vegetative cells are vastly different. For example, only 5 of 89 (approximately 6%) of the transcription factors showing a heat stress response in pollen showed a similar response in vegetative tissues (57). Pollen cells may have evolved novel heat stress responses because they face unique selection pressures. While vegetative cells can respond to a heat stress by slowing down metabolism into a survival mode, an analogous slowdown in pollen might result in a pollen tube losing a competitive race to fertilize a limited number of ovules. Understanding how to improve pollen heat stress tolerance in crop plants is an important goal for food security because climate change is expected to increase the frequency of weather extremes.

SUMMARY POINTS

1. Because pollen are haploid and express nearly half of the *Arabidopsis* genome, researchers should consider testing for mutant phenotypes by quantifying changes in pollen transmission efficiency ratios (TEr).
2. Rho GTPase of plants (ROPs) are small G-proteins that function as molecular switches. In pollen, ROP effectors regulate cytoskeletal dynamics, signaling through reactive oxygen species (ROS), and phosphoregulation of cellular growth machinery.
3. The multiple Ca^{2+} signaling circuits in *Arabidopsis* pollen might involve 39 different Ca^{2+}-permeable channels. The Ca^{2+} circuit(s) that generates a tip-focused Ca^{2+} oscillation could function like a drum beat to synchronize growth processes, for example, by inhibiting ROP-effector-dependent pathways important for pollen tube tip growth.
4. Pollen tube navigation, which is complex and mediated by a series of cell–cell interactions between the pollen tube and the pistil and later between the pollen tube and the female gametophyte, ensures two immotile sperm reach two female gametes and effect double fertilization.
5. Sporophytic tissues in the pistil, including stigma, style, transmitting tract, septum, and ovule integuments, facilitate pollen tube germination, growth, capacitance, and guidance.
6. Synergid cells in the female gametophyte mediate at least three critical signaling events. They are essential to attract the pollen tube into the ovule, receive the pollen tube in a receptive synergid cell (which also undergoes programmed cell death), and induce its explosive lysis, sending a pair of sperm cells to the site where they fuse with female gametes.

7. Released sperm cells are activated and fuse with the two female gametes in the female gametophyte. The mechanism that mediates cell fusion between gametes is ancient and homologous to that used by some enveloped viruses to enter host cells.
8. Each of the signaling events controlling the complex journey of a pollen tube in the pistil is essential for seed crop yield and is potentially temperature sensitive. It is imperative to understand these mechanisms so that we can adapt crop plant productivity to a rapidly changing climate.

DISCLOSURE STATEMENT

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140. Among the first papers to describe pollen tube guidance receptor complexes (also see 132).