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Pollen–pistil interactions: It takes two to tangle but a molecular cast of many to deliver

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Abstract

Explosive advances have been made in the molecular understanding of pollen-pistil interactions that underlie reproductive success in flowering plants in the past three decades. Among the most notable is the discovery of pollen tube attractants [1*,2*]. The roles these molecules play in facilitating conspecific precedence thus promoting interspecific genetic isolation are also emerging [3–5]. Male-female interactions during the prezygotic phase and contributions from the male and female gametophytes have been comprehensively reviewed recently. Here, we focus on key advances in understanding the mechanistic underpinnings of how these interactions overcome barriers at various pollen-pistil interfaces along the pollen tube growth pathway to facilitate fertilization by desirable mates.

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Plant reproduction, Malectin-like domain receptor kinases, FERONIA, GPI-APs, RALF peptides.

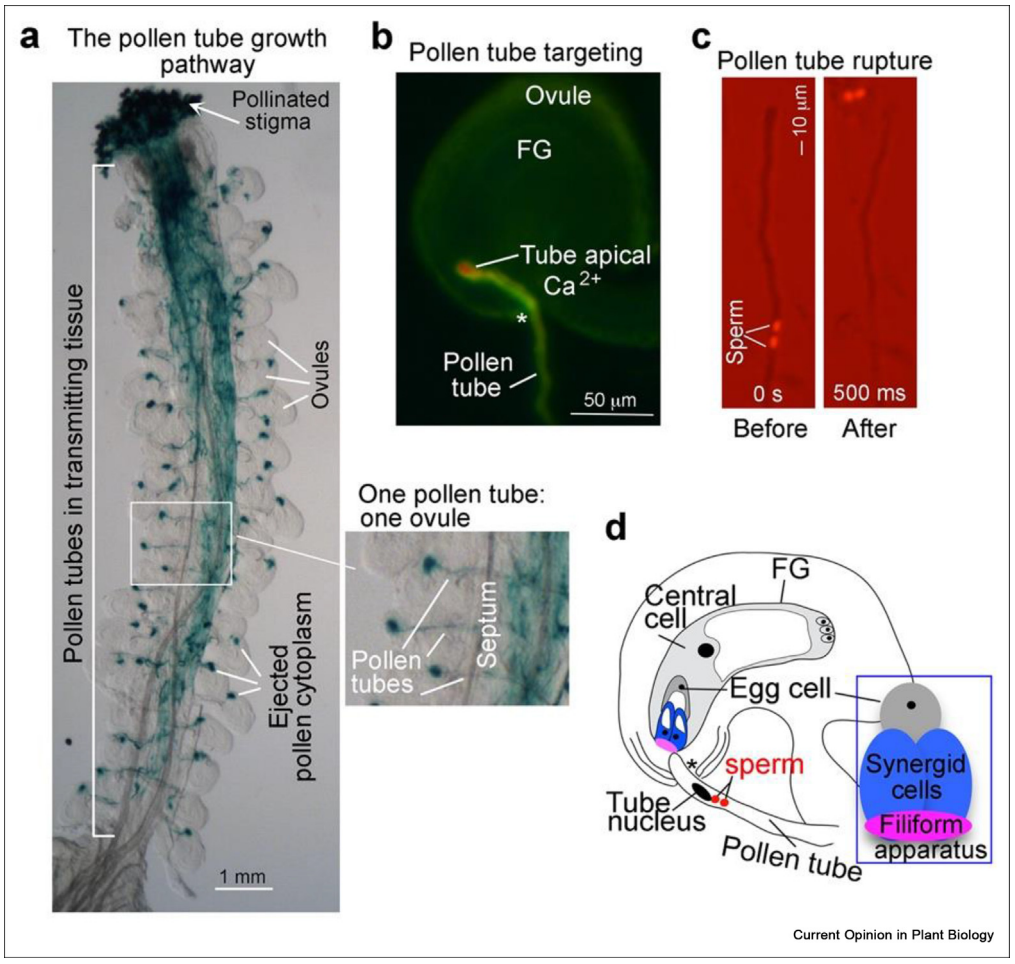
Introduction

Having non-motile sperm, flowering plants depend on sequential male–female interaction events starting from pollen hydration on the stigma to sperm delivery to the female gametophyte inside an ovule for fertilization (Figure 1). Multiple barriers need to be overcome to avert unwanted mates but facilitate desirable mates for eventual encounter and fusion of the male and female gametes. Upon landing on the stigma, the pollen grain hydrates and germinates a pollen tube to penetrate the pistil and transport sperm as its cytoplasmic cargo to the female gametophyte. The pollen tube burrows through the extracellular matrix (cell wall) of pistillate cells before exiting the transmitting tissue in the main pollen tube growth pathway to target ovules in response to pollen tube attractants [1–5] one at a time and penetrate one of the paired synergid cells at the entrance of the female gametophyte (Figure 1). Upon penetrating the female gametophyte, in an event referred to as "pollen tube reception" [6*,7*,8*], the two encapsulated sperm cells are released allowing them access to the female gametes, the egg and the central cells, for fertilization to produce a seed. Multiple barriers have evolved in higher plants to prevent supernumerary pollen tubes from penetrating the same ovule and avoid polyspermy. Research in the past decade elucidated that at the core of these strategies is a molecular signaling triad comprised of a receptor kinase and a glycosylphospatidylinositol-anchored protein (GPI-AP) functioning as a coreceptor pair, and its ligand, a peptide from the rapid alkalinization factor (RALF) family [9,10*,11*]. Each of these signaling triad bears distinct elaborations to fulfill its mission (Table 1). We start with a brief description of the prototypical signaling triad to facilitate the discussion of recent advances in understanding how these signaling modules contribute to reproductive success, derived mostly from studies in Arabidopsis.

A malectin-like domain receptor kinase–glycosylphosphatidylinositol-anchored protein (GPI-AP)–RALF peptide signaling triad

The receptor kinase of the triad is a member of the malectin-like domain receptor kinase family [12*,13*]. In Arabidopsis, seven of the seventeen-membered protein family play important roles in reproduction, three from the pistil and two related pairs from the pollen (Table 1). The coreceptor is from one of four related GPI-APs collectively referred to as the LORELEI

Figure 1



Pollen–pistil interactions. (**a**) The pollen tube growth journey in Arabidopsis. Pollen grains deposited on the stigma hydrate and germinate, each extruding a pollen tube to penetrate the pistil. Pollen tubes elongate in the extracellular matrix (cell wall) secreted by the transmitting tissue. When sensing attractants from ovules [1*,2*], they exit the main growth path, one at a time, to target an ovule and enter the female gametophyte (FG, in **b**,**d**) where the female gametes, the egg and central cells, are located. The blue dots are the ejected pollen cytoplasm, including its cargo sperm cells.(**b**) A single pollen tube at the micropyle (*), an ovular aperture and in the process of growing towards the FG and still maintaining a high apical [Ca^{2+}]. (**c**) An in vitro growing pollen tube immediately before and after tube rupture and sperm release (within 500 msec) under a high ROS condition that mimics the surrounding of the entrance to the female gametophyte. (**d**) A schematic sketch of an ovule, the FG and a penetrating pollen tube with two sperm cells as its cytoplasmic cargo. The inset shows the entrance to the FG, the synergid cells that secrete attractants to guide pollen tube entrance [1*,2*], and the egg cell. The filiform apparatus is a thick cell wall region secreted by the synergic cells and innervated by synergid cell membrane.

(LRE)/LRE-like GPI-AP (LLG) family [14*,15*,16**, 17]. Partnerships assemble between co-expressed receptor kinases and GPI-APs. In the pistil, LLG1 partners with FERONIA (FER) and ANJEA (ANJ) to mediate pollen germination on the stigma [18**], LRE partners with FER, ANJ/HERCULES1 (HERK1) to mediate pollen tube reception [16**,19**,20*]. FER, ANJ and HERK1 also function to assure single pollen tube exits from the transmitting tract and the one pollen tube to one ovule relationship (Figure 1) [21**,22**]. This phenomenon is believed to reduce the chance for polyspermy, fertilization of an egg by multiple sperm cells, which could be adverse for progeny health but could also be beneficial in some cases [23*,24*,25*]. In the pollen, LLG2 and LLG3 partner with ANXUR (AUX) 1 and AUX2, BUDDHA's PAPER SEAL (BUPS)1 and BUPS2 to ensure pollen tube arrival at the ovules [26**,27*,28*]. Regulating the activity of the receptor kinase–GPI-AP complexes are several RALFs, serving as ligands to the coreceptors [10*,18**,22**,26**,27*,28*, 29*,30*].

The malectin-like domain receptor kinases are important players in plant growth, develop and survival [12*,13*]. The pathway that mediate pollen–pistil interactions is best understood for the FER–LRE [19**,21**] and FER–LLG1 [18**] signaling modules. FER functions as a cell surface receptor for the RAC/ROPs (RHO GTPases

Table 1

Malectin-like domain receptor kinase–LORELEI/LLG1 GPI-AP–RALF signaling triad and other key regulatory molecules in prezygotic pollen–pistil interactions.

Prezygotic phase	Signaling module		Signal mediator	Function
	Pistil	Pollen		
Pollen–stigma interaction	FER, ANJ LLG1 RALF23, RALF33	PCP-Bs	RAC/ROPs ROS	Control penetration of pollen into pistil; function is required for compatible pollen germination and arrest of incompatible pollen [16**,32**]
Pollen tube main growth path		AUX1/2 BUPS1, BUPS2 LLG2/3 RALF4/19	ROS MARIS	Maintain pollen tube wall integrity to prevent precocious rupture; function is required upon and shortly after pollen germination [24**,25*,26*,57*,58*]
	FER, ANJ/HERK1	RALF6,7,16 RALF36,37		Control pollen tube exit, implementing polyspermy block after first tube exit to maintain one pollen tube: one ovule pattern [19**,20**]
Pollen tube– ovule/ female gametophyte interaction[a]	FER, ANJ/HERK1 LRE	RALF6,7,16 RALF36,37	RAC/ROPs ROS, Ca^{2+}, NTA/MLO7	Mediates pollen tube reception (sperm release) [17**,20**,40**,44*]
	FER, ANJ/HERK1 LRE	RALF6,7,16 RALF36,37	Pectin, NO	Disengage pollen tube attraction upon first tube arrival, suppress late pollen tube entry into ovules to ensure polyspermy block [19**,20**]
	ECS1, ECS2			Degrade pollen tube attractant upon egg cell fertilization [78**]

[a] Ovule attractant–pollen tube response has been extensively reviewed [1*,2*,82*].

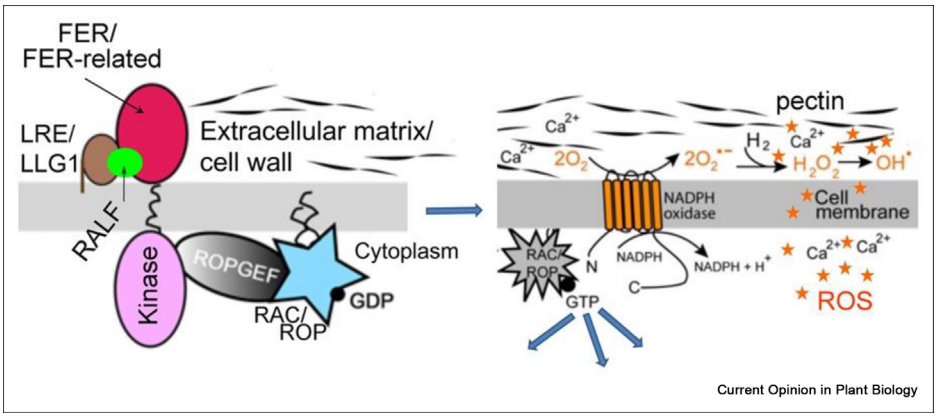
in plants), major molecular switches in plants [31*,32] to mediate NADPH oxidase-dependent production of reactive oxygen species (ROS) (Figure 2) [33**]. FER to ROS signaling regulates growth [16**,33**], controls reproduction [18**,19**,34**], and mediates plant-rhizosphere microbiome interaction [35*]. FER also regulates expansive cytoplasmic networks that regulate metabolic and immunity responses [36,37**]. Additional molecular components and cellular conditions known to function with the FER–LLG1 and FER–LRE signaling in reproduction are summarized in Table 1.

The early breakthroughs: Discovery of FER–LRE in controlling pollen tube reception

Discoveries studying "pollen tube reception" [6*], the last prezygotic step (Figure 1) are instrumental towards today's understanding of pollen–pistil interactions. Under normal conditions, a growing pollen tube terminates at the filiform apparatus (Figures 1 and 3), the cell wall region at the entrance to the female gametophyte. During pollen tube reception, a pollen tube enters a synergid cell and bursts almost instantaneously, ejecting its cytoplasm along with two sperm cells (Figure 1). Several studies identified mutants impaired in this process and their underlying mutations in FER and LRE [14*,15*,38,39,40**]. These mutants are severely female deficient. In homozygous *fer-4* mutant [19**], about 80% of the mutant ovules display a dramatic pollen tube pile-up due to overgrowth (OG) inside the female gametophyte because the penetrated pollen tube has failed to rupture (Figure 3), thus also failing in fertilization. About 40% of *fer* and *lre* ovules show supernumerary pollen tube penetration, the majority of these also displays pollen tube OG. FER and LRE are prominently located at the filiform apparatus [19**,40*,41] and are crucial for maintaining a high ROS environment surrounding the micropyle-filiform apparatus region (Figure 3), a condition required to induce pollen tube rupture in the female gametophyte [19**]. In addition to FER, ANJ and HERK function redundantly to regulate pollen tube reception, double *anj herk* mutants displaying a high level of pollen tube OG and reduced female fertility [20].

The FER–LRE signaling pathway is elaborated by additional molecular and ionic components (Table 1). NORTIA (NTA)/MLO7, a member of the Mildew Locus O [MLO] seven-transmembrane protein family, is another component of the FER–LRE regulated pathway [42*,43]. While FER and LRE localize constitutively at the filiform apparatus [19**,40*,41], NTA translocates from the synergid endomembrane system to the cell membrane at the filiform apparatus in response to pollen tube arrival and this polarization depends on FER. Loss of NTA affects pollen tube reception, although the impact is relatively mild and only ~20% of the mutant ovules display pollen tube

Figure 2



The FER and FER related receptor kinase–LRE/LLG GPI-AP–RALF peptide signaling module and its core signaling pathway. FER was found to interact with ROPGEFs, the guanine nucleotide exchange factors that stimulate exchange of GDP for GTP to activate RAC/ROPs [33*], a major molecular signaling hub in plants [31*,32]. FER partners with LLG1 or LRE, together they function as a coreceptor pair for peptide ligands RALFs [10*,16**,18**,49**] and activate RAC/ROP signaling. NADPH oxidase produces ROS and is a downstream target of RAC/ROPs, among several effectors, together they mediate myriad processes. FER–LLG1/LRE stimulates RAC/ROP-mediated ROS production and the pathway has been demonstrated important for controlling seedling growth [16**,33**], pollen hydration [18**,34**], pollen tube growth [28*] and reception [19**], and plant-rhizosphere microbiome interaction [35*]. Similar modules comprised of pollen-expressed counterparts of FER, LLG and RALFs are important for pollen tube integrity during growth in the pistil [26**,27*,28*], also depends on NADPH oxidase and a regulated ROS environment [28*,59*,60*].

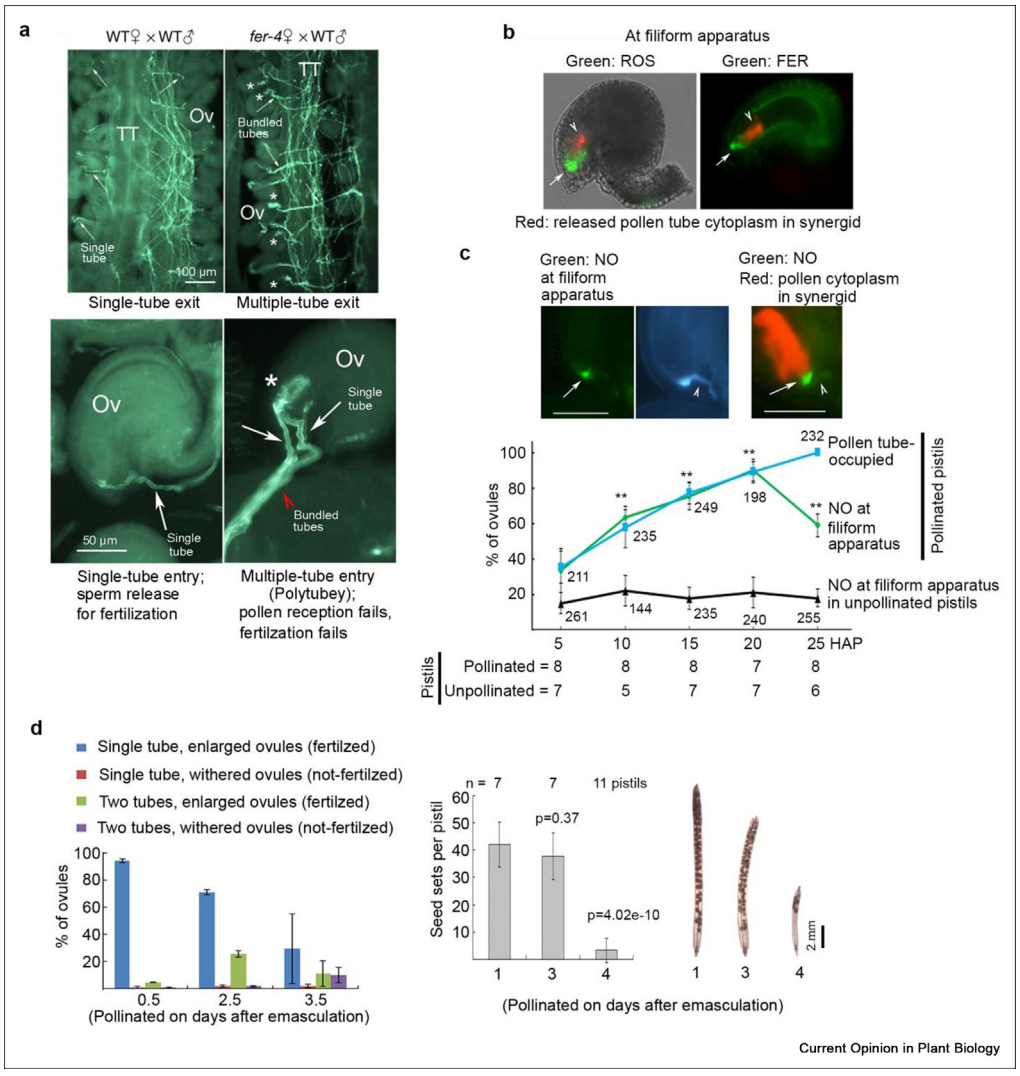
OG. These together led to the suggestion that NTA/MLO7 potentially acts as a signal booster to ensure pollen tube rupture [42*,43].

In addition to the ROS maximum at the entrance of the female gametophyte, a certain threshold level of Ca^{2+} is required for pollen tube bursting [19**]. Cytoplasmic [Ca^{2+}] in the synergids is impacted by the approaching pollen tube, which induces oscillatory Ca^{2+} spikes and the response is dependent on FER, LRE and NTA [44–46]. The extracellular [Ca^{2+}] condition at the filiform apparatus has to be lower than the threshold needed for pollen tube bursting, otherwise it would be detrimental to fertilization. Nevertheless, the FER-conferred high ROS condition at the filiform apparatus could have weakened the pollen tube wall integrity in some still unknown ways, priming it for bursting once inside a synergid. The spatial separation of the ROS maximum and pollen tube bursting suggests that a [Ca^{2+}] threshold is met by the pollen tube only after it has grown past the filiform apparatus and entered a synergid. NTA/MLO7 has a C-terminal located calmodulin-binding domain, characteristic of MLO proteins and therefore can potentially participate in the pollen-triggered Ca^{2+} signaling events during pollen tube reception [42**,43,47]. A recent study identified several pollen-expressed RALFs, RALF6, 7, 16, 36 and 37, as important for pollen tube reception [22**]. Pollination by double *ralf36, 37*, and quintuple *ralf6, 7, 16, 36, 37* mutants induces high levels of ovules displaying pollen tube OG. These pollen RALFs bind to the extracellular domain of FER, ANJ and HERK1 *in vitro*, and RALFs are known to trigger Ca^{2+} influx [48,49**]. Taken together, these pollen RALFs could be strong contenders as the signals [22**] that trigger the pollen tube-induced FER-, LRE- and NTA-dependent synergid Ca^{2+} response [44,45,46].

Pollen-stigma interaction: Opening the stigmatic gate for a desirable mate

The stigmatic papilla of finger-like cells provides a barrier for unwanted penetration and stigmatic redox condition [50–52] plays a crucial role in maintaining this gate [18**,34**]. In Arabidopsis, FER– and FER/ANJ–LLG1–RALF23/33 signaling modules (Table 1) maintain a tunable level of ROS in the mature receptive stigma papillae via the RAC/ROP-mediated NADPH oxidase pathway (Figure 2) [18**]. The deposition of compatible pollen triggers a reduction of stigmatic ROS, which is crucial for pollen hydration and germination of a pollen tube to penetrate the stigmatic papilla [18**,34**]. Pollen hydration on mutant stigmas lacking one of the components of the signaling triad, or with down-regulated RAC/ROPs or NADPH oxidase is accelerated significantly than on wild type stigmas, discernible as early as two minutes after pollen deposition. Pollen tubes also reach notably longer distances in the transmitting tract during the early hours after pollination relative to in the wildtype pistil. Several POLLEN COAT PROTEIN B-class peptides (PCP-Bs) [53] function as triggers to disengage the stigmatic FER- and ANJ-regulated ROS pathway. PCP-Bs interact with FER

Figure 3



Pollen tube reception and polyspermy blocks: the FER perspective Pollinated wild type (WT) and loss-of-function *fer* pistils. Studies of *fer* mutants reveal the role of FER in an early polyspermy block in the main pollen tube growth pathway and a local block at the ovule [21**,22**], and its role in pollen tube reception [18**,38,39,40*]. (**a**) In pollinated *fer-4* pistils, multiple pollen tubes exit the transmitting tract in bundles and penetrated single ovules in bundles, but fail to rupture in the female gametophyte, resulting in a pollen tube pile-up (*). From the study by Duan et al. [21**]. "Polytubey" has also been used to refer to this phenotype, though mechanistically it different from how "polytubey" is induced under "fertilization recovery" conditions [85**,86**,87**]. (**b**) Two ovules after pollen tube reception showing filiform-located FER (right) maintains a ROS maximum surrounding the filiform apparatus (left) with the ejected cytoplasm from the received and ruptured pollen tube to highlight the spatial relationship between FER, ROS and the pollen tube reception event. From the study by Duan et al. [19**]. (**c**) [Image panel] Pollen tube (arrowhead) arrival induces NO accumulation at the filiform apparatus [arrow]. The right panel shows a merged image of an ovule with NO at the filiform apparatus and the penetrated synergic cell filled with the ejected pollen tube cytoplasm. [Plot] Data showing the coincidental arrival of pollen tube and the appearance of NO at the filiform apparatus in pollinated pistil. From the study by Duan et al. [21**]. The multiple pollen tube exit from the transmitting tract and their bundled entrance phenotype closely tracked the pollen tube arrival time course [21**,22**]. (**d**) An "aging mommy's" strategy to prolong fertility. Pollination of aging pistils (pollinated at 3.5–4 days after emasculation or just pre-anthesis pistil) when ovules were past prime reveal delayed implementation of the polyspermy block at the ovule, allowing multiple pollen tube penetration (green and purple bars) [left panel], increasing the chance that one might rupture even under declining female gametophyte condition so that a low level of seed set is salvaged [right panel]. Data was obtained by Q-h Duan, assisted by Laura Gates (University of Massachusetts).

and ANJ with higher affinity than RALF33, presumably competing with RALF33 to interact with the signaling triad thus downregulating ROS production, thereby disengaging the stigmatic gating mechanism [18**]. FER homolog from the self-incompatible *Brassica rapa* functions in a similar capacity [34**]. Not only that the *B. rapa* FER-regulated stigmatic ROS is tuned down by arriving cross-compatible pollen allowing for their

hydration on the stigma, self-incompatible pollen triggers the opposite effect, elevating stigmatic ROS to block their germination.

Pollen-assembled receptor kinase–LLG–RALF signaling triads ensure a successful pollen tube journey to ovules

The pollen tube is an autonomous growth engine, and voluminous reviews have been written for these cells grown in simple culture media [54**,55,56*]. The highly polarized pollen tube has a relatively malleable cell wall at the apex to allow rapid tip-growth and it stiffens when displaced to distal regions of the elongating tube as the tip advances. Maintaining pollen tube integrity when growing in the pistillate extracellular matrix is the task of pollen-produced signaling modules comprise of ANX/BUPS receptor kinase heteromers, LLG2/3 and RALF4/19 [26**,27*,28*] (Table 1). ANX1 and 2 function redundantly and double *anx1 anx2* mutants are male sterile [57,58] whereas BUPS1 almost exclusively carries the functional burden for the BUPS pair [26**]. In culture, many *anx1 anx 2*, *bups1*, and *bups1 bups2* pollen grains burst almost immediately upon germination, and a notable percentage of the germinated tubes burst during growth, consistent with a considerably weakened tube wall. In the pistil, mutant pollen tubes almost never reach an ovule but growth-arrested presumably have burst, prior to entering the ovary, hence the male sterility. *llg2 llg3* and *ralf4 ralf19* pollen tubes both burst precociously and the mutant plants are male sterile [26**,27*,28*,30*], consistent with their being part of the same signaling modules. Downstream signaling components of these pollen tube-assembled signaling triads include NADPH oxidase and ROS [28*,59*], and a receptor-like cytoplasmic kinase, MARIS, involved in maintaining cellular ROS homeostasis [60*]. Therefore, these signaling modules ensure pollen tube integrity by maintaining a tip growth-supporting level of pollen tube ROS during its journey in the pistil. The ovule RALF34 competes with RALF4 and 19 in binding to ANXs and BUPSs *in vitro* [26**], this and other ovule-expressed RALFs might together contribute to mediating pollen tube reception.

Polysaccharide and protein components of the cell wall are abundant and should contribute to its integrity. Leucine-rich repeat extensin (LRX) proteins are conserved across monocots and dicots, many of them are expressed in pollen and tightly associated with the cell wall [61–63,64*]. In Arabidopsis, four pollen-specific LRXs interact with RALF4 and RALF19 [30*,65]. Multiple combinatorial mutants in these LRXs show myriad pollen phenotypes, ranging from pollen germination, tube growth, changes in pollen tube wall properties and reduced seed sets [66,67]. In the pistil, fewer *lrx* mutant pollen tubes reach ovules than normal and they also have a high propensity to burst in culture. These together reveal a node where LRX interaction might intersect with the ANX/BUPS–LLG2/3 regulated pathways to impact pollen tube growth. Functional and molecular interactions have been demonstrated between seedling-expressed LRXs and the FER signaling module to mediate stress responses [68–70]. How pollen LRXs–RALF14/19 interactions [30*] integrate with ANX/BUPS–LLG2/3–RALF4/19 [26**,27*,28*] signaling remains to be determined.

The mysterious polyspermy blocks: How to close an open door?

Polyspermy blocks in flowering plants are complicated and have been a subject of long-standing interest [23*,24*,25*,71*,72*,73*]. There are multiple check points, therefore mechanisms for their implementation and relaxation are likely to be multi-faceted. To implement a polyspermy block relies on the pistil sensing fertilization has been assured so it can safely turn a receptive environment into a blockade; the timing would seem crucial. Relaxing these blocks is also important and relies on the female gametophyte sensing imminent danger of being left unfertilized so as to trigger mechanisms to prolong its window for fertility. In the Arabidopsis pistil, pollen tubes can clearly be traced as predominantly exiting the main growth path one at a time to maintain the one pollen tube:one ovule pattern (Figures 1 and 3). Late-arriving pollen tubes are not attracted to approach an already penetrated ovule deterring them to other not yet fertilized ones [74,75], thus reducing the chances for polyspermy and maximizing reproductive success. The dual phenotype of multiple pollen tube entries and OG at the *fer* and *lre* mutant ovules and in female gametophytes, respectively (Figure 3) [14*,15*,19**,38,39] support close intimacy between pollen tube reception and implementation of a polyspermy block, although the two processes are not obligatorily coupled [19**].

However, to maintain the one tube:one ovule pattern, a critical check point clearly occurs considerably earlier when pollen tubes exit the main growth path (Figure 1). In fact, FER already plays a crucial role at this early block since many pollen tubes exit in bundles in *fer* mutant pistils [21**], thus setting the stage for multiple pollen tubes penetrating an ovule in bundles (Figure 3a). ANJ and HERK1 also function redundantly, irrespective of the presence of FER, in blocking late-coming pollen tubes [22**]. More importantly, this recent study also discovered that the five pollen-expressed RALFs, 6, 7, 16, 36 and 37, which are important for triggering pollen tube reception at the ovule, also collectively ensure the implementation of the pollen tube block at the main growth pathway. Loss of these RALFs induces bundled pollen tube exits from the transmitting tract and their entry into ovules, similar to phenotypes observed in *fer* and *anj herk1* pistils

[21**,22**]. These peptides are therefore the likely pollen triggers that locally signal FER and ANJ/HERK1 to implement a gate, closing immediately after the first pollen tube has exited the transmitting tract and again later when the tube has gained access to the ovule. Precise mechanisms of how these pollen-expressed RALFs work remain to be determined.

Local blocks at the ovule [74] against late-arriving pollen tubes are likely an important insurance to further safeguard against errand pollen tubes that have evaded the earlier polyspermy block along the main growth pathway. A three-step mechanism mediated by fertilization to block late-arriving pollen tubes has been proposed [76*,77*], the first being an early but temporary mechanism involving weakening of pollen tube attraction; this is supported by more recent studies [21**,78**]. This is followed by what appear to be two interconnected processes, one dependent on components of the ethylene signaling pathway but not ethylene per se, the other involving chromatin re-structuring [79*,80*,81*]. These together would induce death and therefore elimination of the uninvaded, and so called "persistent," synergid cell, thus obliterating any chance for a source of attractants to incentivize late-approaching pollen tubes.

Pollen tube-pistil cell wall interaction plays an important role as sensor and mediator of the FER-mediated polyspermy block at the ovule [21**]. Pollen tube growth is through the wall secreted by pistil cells [82] and FER binds the cell wall polysaccharide pectin impacting cell wall property [83**], including that of the pistil [21**]. The wild type pistils maintain an extracellular matrix rich in de-esterified pectin, including in the transmitting tract and at the filiform apparatus, and *fer* mutant pistils are deficient in this cell wall polysaccharide. A series of experiments established a FER to nitric oxide production pathway via FER-dependent de-esterified pectin at the filiform apparatus as important for maintaining the ovular block for multiple pollen tube entries (Figure 3c). It also showed that nitric oxide acts in a two-pronged mechanism to disengage the pollen tube attraction machinery, one by inactivating the already secreted pollen tube attractant and the other inhibiting its secretion [21**].

It is never too much to be safe. Even after the FER-dependent and pollen tube arrival-triggered blockade acting from the filiform apparatus region, a post-fertilization mechanism initiated from the fertilized egg provides additional measures to ensure against supernumerary pollen tube penetration of the same ovule [78**]. The study showed that successful fertilization triggers secretion from the egg endopeptidases (ECS1 and ECS2) to degrade pollen tube attractant AtLURE1 [84**], thus further removing any residual attractant for late-approaching tubes. A low but significantly higher percentage of *ecs1 ecs2* ovules than normal display the multiple pollen tube phenotype, reflecting continued attraction of pollen tubes by these mutant ovules.

An aging Mommy's strategy: "Fertilization recovery" induces "polytubey" to salvage seed production

Nature's strategies for species survival are abundant and elegant. One of these is deployed when fertilization fails in flowering plants to at least salvage some seed formation to ensure species survival. The phenomenon was systematically examined in Arabidopsis pistils pollinated by the *hapless2-1/generative cell-specific1*mutants whose sperm cells fail to fuse with the egg. The strategy is referred to as "fertilization recovery" [85**,86**], and the consequence is that multiple pollen tubes are allowed to access the ovule, hence the term "polytubey" was coined as reflected by the phenotype [87**]. Although phenotypically similar, hallmark distinctions between "fertilization recovery"-induced "polytubey" [85**,86**,87**] and the multiple pollen tube entries induced by compromised polyspermy blocks [21**,22**,78**] are in their timing and the unfulfilled pollen tube- or sperm-mediated events that trigger them. The compromised polyspermy block-induced phenotypes along the pollen tube growth pathway and at the ovules in *fer*, *anj/herk1* pistils and in wild type pistils pollinated by *ralf6,7,16,26,37* start at about 5—6 h after pollination, coinciding with or just slightly trailing the earliest pollen tube arrivals at the ovules [21**,22**]. This timing reflects an abrupt transition from an open door for the first-arrived pollen tube to a closed gate for late-comers under normal condition. Microscopic observations demonstrate that the loss-of-female gametophytic FER- and loss-of-pollen RALFs-induced multiple pollen tube phenotype is a result of sperm access to the egg being physically blocked by remaining trapped inside the overgrown pollen tube [19**,20,40*].

In *ecs1 ecs2* ovules, the second pollen tube should have entered by 6 h after pollination since two pairs of sperm cells can be observed between 6 and 8 h in some of the mutant female gametophytes [78**]. Observations of sperm arrival in the female gametes and normal rates of fertilization in *ecs1 ecs2* mutant ovules established a post-sperm release mechanism. They also imply successful sperm—egg fusion and that a post-fertilization event underlies the weakening of the block against multiple tube entrance. The timing is also consistent with the female gametophyte allowing time for fertilization to take place prior to removing any residual incentive for more pollen tube penetration. The relatively low percentage of *ecs1 ecs2* ovules displaying multiple pollen tube entry [78**] is also consistent with ECS1 and

ECS2 being dispatched by the fertilized egg as further insurance after the FER- and ANJ/HERK1-controlled and pollen tube arrival-triggered mechanism has already decapacitated the attraction process [21**].

By comparison, "fertilization recovery"-induced "polytubey" by gamete fusion mutants [85**,86**,87**] occurs relatively late, readily observed only starting around 8–12 h after pollination and continues to increase till 16–24 h after pollination [21**,22**,86**]. The clear presence of sperm in the female gametophytes supports a post-sperm release mechanism. This, together with the sperm cells being defective in fusion with the egg, implies a mechanism triggered by a failure in sperm–egg membrane fusion in the absence of fertilization. The relatively late time frame is consistent with allowing time to re-activate a program for pollen tube targeting by maintaining viability of the "persistent" synergid to regenerate a pollen tube attractant gradient [76*,77*,79*,80*]. It also requires the reopening of already shut polyspermy gates [21**,22**], as well as suppressing ECS1 ECS2 [78**]. How to reverse the blocks for secondary pollen tubes is intriguing, possibly even more complex and multifaceted than how these barriers are established. The pollen RALF-signaled FER- and ANJ/HERK1-mediated pollen tube reception at the ovules and the consequential rapid dissipation of pollen tube RALFs along the shank of the burst tube has been suggested a potential link to relieve the polyspermy block at the main growth path for "fertilization recovery" [22**].

Though observed in nature but considered anomalous [86**], the "polytubey" phenotype and the need to salvage from lost opportunities for fertilization is likely more commonplace than apparent. In particular, when under conditions adverse to reproduction, for example, weather calamities or the absence of compatible pollen when the ovules are at their prime, prolonging the window of opportunity for fertilization would seem prudent. As shown in Figure 3d, ovules from past-prime pistils are prone to display "polytubey" when pollen finally arrives, salvaging at least a low level of seed formation. Mechanistic models for polyspermy block implementations, their relaxation or reversal, and the activation of additional programs to prolong the window of opportunity to produce progeny [e.g., the studies by Duan et al., Zhong et al., Maruyama et al., Maruyama et al., Yu et al., Völz et al., and Maruyama et al. [21**,22**,76*,77*,78**,79*,80*] will likely undergo rounds of refinements and recalibrations for precise understandings to emerge.

Outlook

Gene discoveries, biochemical and cell biological studies have considerably advanced the mechanistic understandings of various pollen–pistil interaction steps that enable fertilization, but many questions remain. The actions of active oxygen species are varied for distinct stages of the long pollen tube journey in the pistil journey [18**,19**,21**,27*,34**,59*,60*]. The various pollen responses underscore the importance of the diverse functional roles of ROS in controlling processes from cell growth to cell death [88*]. Investigating the biochemical and cellular consequences of ROS actions in the various pollen and pollen tube responses should provide insights on how the different processes are mediated. The stage is set for diving deeper into many remaining mechanistic puzzles. For example, how is the pollen hydration and tube extrusion processes initiated? What does it take to maintain pollen tube wall extensibility and elastoplasticity to sustain its growth inside the pistil? What are the threshold levels of ROS and Ca^{2+} on either side of the pollen tube-synergid cell interface required for sperm release? How does an approaching pollen tube trigger the needed Ca^{2+} response in the synergids to induce rupture of the penetrating pollen tube? How are the oxidative and ionic conditions maintained to ensure pollen tube integrity during its passage through the filiform apparatus and induce its rupture almost instantaneously upon entry of a synergid?

On the molecular level, the story of whether and how ovular RALFs contribute to inducing pollen tube reception remains unfinished. The stories of the PCP-Bs on the stigma, and pollen RALFs interacting with female signaling modules prompt questions on whether there are more of male-female interactive partners of similar designs as well as those comprised of different molecules. With players involved in implementing polyspermy blocks being uncovered and post-fertilization events being elucidated, targets are becoming available to contemplate how these blocks may be relaxed or delayed and, when fertilization fails, how "fertilization recovery" is activated. Understandings of the diverse strategies utilized by flowering plants to facilitate fertilization may be brought to bear in future efforts to manage fertility and improve seed yield and quality, impacting food sources and plant diversity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Through the use of mutants with sperms defective in fusion with the female gametophyte, this paper uncovered an elegant strategy in Arabidopsis, appropriately termed “fertilization recovery,” that salvages reproduction in the face of failed fertilization.

86. ** Kasahara RD, Maruyama D, Higashiyama T: **Fertilization recovery system is dependent on the number of pollen grains for efficient reproduction in plants**. *Plant Signal Behav* 2013, **8**:e23690.

87. ** Beale KM, Leydon AR, Johnson MA: **Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule**. *Curr Biol* 2012, **22**:1090–1094.
Through the use of mutants with sperms defective in fusion with the female gametophyte, this paper uncovered the elegant strategy in Arabidopsis, termed “fertilization recovery” in [85**], and coined the phenotype of allowing secondary pollen tube entries “polytubey” induced under conditions of the first-arrived pollen tubes failed in fertilization.

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