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Update

Update on signaling pathways regulating polarized intercellular communication in Arabidopsis reproduction

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Introduction

Cell polarity refers to the asymmetric distribution of cellular components, structure, and function within a cell. Most instances of cell polarity in plants involve single cells or groups of cells that are attached to neighboring cells by the cell wall. Flowering plant reproduction is unique because it involves intercellular signaling between a highly polarized cell, the tip-growing pollen tube, that must navigate its way through various sporophytic tissues of the pistil to find the ovules and deliver its sperm cell cargo to the female gametes deep within the pistil where double fertilization occurs to form the next generation (Johnson et al. 2019). The pollen tube's journey involves a series of cell polarity changes in single cells occurring over short time periods in response to signals from another cell type. These interactions occur between cells that have different ploidy and potentially different genotypes if the pollen comes from a different plant. The haploid male gametophyte, or pollen grain, is formed in the anther and is the product of meiosis followed by 2 rounds of mitosis to form the vegetative cell with 2 embedded sperm cells (Fig. 1) (Hafidh and Honys 2021). In most angiosperms, the haploid female gametophyte, also known as the embryo sac, is formed in the ovule and is the product of meiosis followed by 3 rounds of mitosis to form a 7-celled structure that contains 2 gametes, the egg and central cell, and accessory cells known as the synergids and antipodal cells (Fig. 1) (Hater et al. 2020). The goal of pollination is to deliver the 2 sperm cells from the male gametophyte to the 2 gametes in the female gametophyte for double fertilization (Johnson et al. 2019). This goal requires interactions between the pollen grain and a diploid, sporophytic stigma papilla cell, followed by interactions between the tip-growing pollen tube and the sporophytic cells of the stigma, style, transmitting tract, septum, and funiculus (Fig. 1). Finally, the pollen tube interacts with the highly polarized haploid synergid cells of the female gametophyte and gets the signal to release the sperm cells so that polar interactions can occur with the egg and central cell. These interactions between the different cell types result in changes in polarity and growth direction of the tip-growing pollen tube in response to polar signals from the maternal sporophytic and gametophytic cells. (See Box 1 for an overview of tip growth in pollen tubes.) A recurrent theme in these intercellular communication events is the perception of peptide ligands by polarly localized receptor-like kinases (RLKs) leading to rapid cellular events. At several stages of plant reproduction, the CrRLK1L family of malectin-like RLKs respond to RAPID ALKALINIZATION FACTOR (RALF) or other peptide ligands secreted from a different cell type (paracrine signaling) or from the same cell (autocrine signaling) to trigger signal transduction cascades that regulate pollen tube growth direction and integrity (Zhu et al. 2021). In this Update, we will discuss the latest advances in understanding the regulation of polarized pollen tube growth and intercellular signaling during plant reproduction, with an emphasis on research from the model plant Arabidopsis (*Arabidopsis thaliana*).

Establishing polarity in the pollen grain and stigma papillae

The male gametes are enclosed within the cytoplasm of the vegetative cell of the pollen grain, which is protected by a tough pollen cell wall (Hafidh and Honys 2021). This allows pollen grains to survive the harsh environments that they

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ADVANCES BOX

- A conserved signaling module consisting of RALF peptide ligands binding to a CrRLK1L and LRE/LLG receptor-complex activating a downstream MLO Ca^{2+} channel is used by the pollen tube to maintain tip growth and by the female tissue to manipulate pollen tube growth.
- During pollen germination, a rapid influx of Ca^{2+} into the pollen grain is mediated by RALF4/19 and the ANX1/2, BUPS1/2, LLG2/3 receptor complex activating pollen Ca^{2+} channels.
- An early polytubey block in the septum is established when pollen tube–secreted RALF6/7/16/36/37 binds to a FER/ANJ receptor complex on the septum. Signaling downstream of FER/ANJ prevents additional pollen tubes from targeting an ovule.
- MLOs were recently identified as a new family of Ca^{2+} channels. In both pollen tubes and synergids, MLOs are activated through CrRLK1L-mediated signaling pathways.

encounter between anther dehiscence and deposition on the stigma by pollinators, wind, or self-pollination through close proximity of the anther and stigma. The first challenge of plant reproduction is to initiate directional pollen tube growth from a dormant pollen grain so that the pollen tube can start its journey toward the female gametophyte. This process involves polarity changes in both the pollen grain and the stigma papillae cell that interacts with that pollen grain (Kandasamy et al. 1994).

Upon interaction with a compatible stigma, the pollen grain undergoes rapid hydration and establishment of polarity that leads to emergence of a tip-growing pollen tube within 20 min in Arabidopsis (Kandasamy et al. 1994). RLK signaling is involved in the stigma's perception and communication with the pollen grain to promote rapid hydration. The CrRLK1L proteins, FERONIA (FER) and ANJEA (ANJ), act to regulate reactive oxygen species (ROS) accumulation in stigma papillae cells which in turn regulates the rate of pollen hydration (Liu et al. 2021). Before pollination, stigma-produced RALF33 interacts with the FER/ANJ receptors to promote ROS accumulation in the papillae cell walls through autocrine signaling. Several small cysteine-rich peptide encoding genes known as POLLEN COAT PROTEIN B (PCP-B) have been implicated in pollen hydration (Wang et al. 2017). A recent study linked 1 of these peptides to RLK signaling in the stigma. During pollen–stigma interactions, pollen PCP-Bγ displaces RALF33 from the receptors and downregulates FER/ANJ-mediated ROS production and the area of the papillae cell wall near the pollen grain (Liu et al. 2021). The mechanism through which decreased ROS in the pollen–stigma interface promotes rapid pollen hydration remains to be discovered. However, the finding that *pcp-Bγ* mutant pollen grains can still hydrate and germinate pollen tubes on wild-type stigmas indicates that ROS regulation in the stigma is not the only determinant of pollen hydration.

Stigma-expressed Leucine-Rich Repeat (LRR) receptor-like kinases have also been implicated in regulating pollen

Box 1.

Pollen tubes are highly polarized tip-growing cells. The pollen tube is a highly polarized tip-growing cell that needs to navigate the pistil to deliver the 2 sperm cells to the embryo sac. Pollen tube elongation requires a delicate balance of speed as pollen tubes that grow too quickly can burst from loss of cell wall integrity and could miss guidance cues secreted from the pistil, but pollen tubes that grow too slowly fail to fertilize ovules (Johnson et al. 2019). The features of pollen tube tip growth have been extensively reviewed (Mollet et al. 2013; Ge et al. 2019a; Johnson et al. 2019; Adhikari et al. 2020; Hayashi and Palmgren 2021; Ou and Yi 2022). Pollen tube elongation occurs at the apical region where exocytosis of vesicles drives plasma membrane expansion (Adhikari et al. 2020). This region is differentiated from the shank of the pollen tube by cell wall modifications including demethylesterification of pectin in the shank that strengthens the cell wall (Mollet et al. 2013; Adhikari et al. 2020). ROP1 functions as a major regulator of polarized exocytosis in pollen tubes and is activated by the RLKs PRK2/6 and ANX1/2 BUPS1/2 that bind to synergid secreted LUREs and pollen tube-secreted RALFs, respectively (Ou and Yi 2022). ROP effectors regulate polarized growth through regulation of the actin cytoskeleton, tip-focused Ca^{2+} oscillations, and ROS generation (Johnson et al. 2019; Ou and Yi 2022). Pollen tube Ca^{2+} channel mutants exhibit a range of phenotypes including altered Ca^{2+} oscillations, impaired pollen tube growth, and pollen tube discharge defects (Ge et al. 2019b; Johnson et al. 2019). ROS homeostasis is also important for maintaining pollen tube integrity during growth through the transmitting tract and may also contribute to pollen tube bursting in the embryo sac (Ge et al. 2019b). In addition, H^+ oscillations at the pollen tube apex play a role in regulating cell wall plasticity and the actin cytoskeleton (Hayashi and Palmgren 2021). The regulation of pollen tube tip growth is crucial for reproduction as these signaling mechanisms allow for directional changes as the pollen tube perceives attractants, pauses in growth during pollen tube reception, and the eventual loss of integrity as the pollen tube bursts.

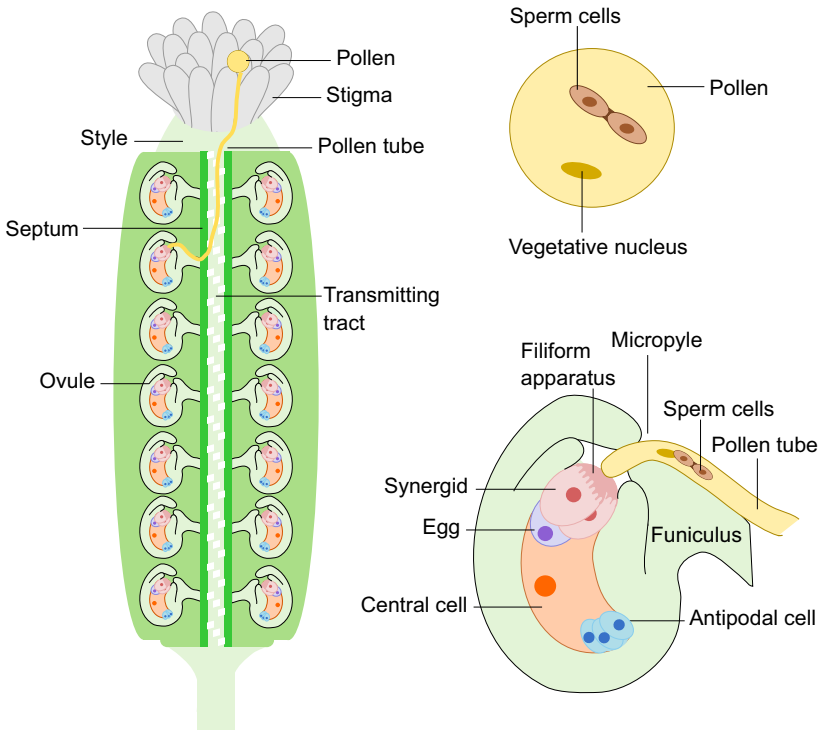

Figure 1. The process of reproduction in Arabidopsis. The male gametophyte is pollen, which contains a vegetative cell and 2 sperm cells. The female gametophyte is a seven-celled structure called the embryo sac containing an egg cell, central cell, 2 synergids, and 3 antipodal cells. A pollen grain lands on a stigma and germinates a pollen tube carrying 2 sperm cells. The pollen tube grows into the stigma through the style and transmitting tract before turning towards an ovule and exiting the transmitting tract at the septum. The pollen tube continues to grow along the surface of the funiculus towards the micropylar end of the ovule as pollen tube attractants are secreted from the synergid cells. Signaling known as pollen tube reception between the receptive synergid and pollen tube mediates the pollen tube's entry into the embryo sac and bursting, fertilizing the egg and central cell.

hydration in compatible pollinations in Arabidopsis. The RKF family of 4 stigma-expressed LRR-RLK genes acts redundantly to positively regulate pollen hydration (Lee and Goring 2021). The RKF proteins have not been studied as extensively as the CrRLK1L family. Neither the ligands nor downstream signaling outputs are known. It will be interesting to determine if these proteins are also involved in regulating ROS during pollen–stigma interactions.

Pollen hydration triggers a rapid influx of Ca^{2+} into the pollen grain and cytoplasmic reorganization that leads to pollen tube emergence (Iwano et al. 2004). In vitro pollen germination experiments in a recent study revealed that this rapid influx of Ca^{2+} may be mediated by receptor-like kinase proteins from the CrRLK1L family, ANXUR1/2 (ANX1/2) and BUDDHA PAPER SEAL1/2 (BUPS1/2), in a complex with the GPI-anchored proteins, LORELEI-LIKE GENE2/3 (LLG2/3), that recognizes pollen-produced cysteine-rich RALF4/19 ligands to activate calcium channels (Gao et al. 2023). These pairs of genes act redundantly with pollen phenotypes only evident in double mutants. *ralf4/19*, *anx1/2*, *bups1/2*, and *llg2/3* mutants all fail to establish the polarized Ca^{2+} gradient in hydrating pollen grains, and emerging pollen tubes are not able to maintain tip growth and burst soon after germination (Boisson-Dernier et al. 2009; Miyazaki et al. 2009; Ge et al. 2017, 2019b; Mecchia et al. 2017; Gao et al. 2023). ANX1/2 and BUPS1/2 are all polarly localized at the tips of growing pollen tubes (Boisson-Dernier et al. 2009; Ge et al. 2017). Two cargo-sorting AP180 N-terminal homology domain-containing proteins (PICALM5a and PICALM5b) are necessary for ANX1/2 localization in the pollen tube tip and the subsequent maintenance of tip growth (Muro et al. 2018). RALF4/19 also regulate pollen tube growth through interactions with LEUCINE-RICH REPEAT EXTENSINS (LRX) that regulate composition of the pollen cell wall (Mecchia et al. 2017; Fabrice et al. 2018; Sede et al. 2018; Wang et al. 2018). Interestingly, RALF4/19 peptides are able to bind pollen tubes at the tip as well as along the length of the pollen tube (Mecchia et al. 2017), possibly reflecting additional roles in determining cell wall composition in other areas of the pollen tube. Consistent with this idea, the RALF4/19-LRX module is able to act independently from the ANX1/2 signaling pathway that occurs at the pollen tube tip (Franck et al. 2018).

Entering the style and transmitting tract

The next polarity challenge during the pollen–stigma interaction phase is to ensure that the pollen tube grows into

the transmitting tract so that it can make the journey to the ovules. In Arabidopsis, the newly formed pollen tube invades the cell wall of the papillae cell and grows down to enter the transmitting tract (Kandasamy et al. 1994). This process requires polarized loosening of the papillae cell wall, a process that is not well understood at the molecular level. This process is tightly controlled in wild-type pollinations under optimal conditions, and most pollen tubes take the shortest path through the loosened papillae cell wall. However, as stigmas age past their prime receptivity stage, changes in papillae wall stiffness are correlated with altered pollen tube behavior in which invading pollen tubes coil around the papillae cell instead of taking a straight route to the transmitting tract. This phenotype is also found in *katanin* mutants, revealing a link between cytoskeletal remodeling and regulation of papillae polarity to guide pollen tube growth (Riglet et al. 2020).

After communication with the stigma, pollen tubes encounter 2 different environments: the style and the transmitting tract. In Arabidopsis, the style consists of tightly packed cells, whereas the transmitting tract cells break down to create a more open pathway in mature (Stage 14) pistils (Crawford et al. 2007; Crawford and Yanofsky 2011). Thus, during the transition from the style to the transmitting tract, growing pollen tubes must recognize and respond to changes in the mechanical pressure exerted from the external environment in order to maintain tip integrity. Artificial microRNA downregulation of BUPS1 or RALF4/19 led to premature bursting as the pollen tube transitioned from the style to the transmitting tract, indicating that these signaling molecules have a mechano-transduction function during pollen tube entry into the transmitting tract (Zhou et al. 2021).

Navigating the transmitting tract to find the ovules

Pollen tube tip growth through the reproductive tract is thought to be supported by nutrients supplied by the pistil (Lennon et al. 1998). During growth through the stigma, style, and transmitting tract, pollen tubes respond to signals from the pistil by expressing genes that lead to capacitation, or the ability to respond to signals from the ovule so that pollen tube attraction and reception can be completed (Palanivelu and Preuss 2006). Transcriptomics experiments comparing in vitro germinated pollen tubes with semi-in vivo pollen tubes that have grown through the stigma and style of a pistil before emerging on pollen germination media identified a few hundred genes that are specifically upregulated during pollen tube growth through the stigma and style (Qin et al. 2009; Leydon et al. 2013, 2017). Reverse genetics approaches identified 3 MYB transcription factors (MYB97/101/120) that are necessary for preparing the pollen tube for reception at the synergids (Leydon et al. 2013; Liang et al. 2013). More recently, this dataset was used to identify candidate genes involved in regulating pollen tube exit from the transmitting tract. In wild-type Arabidopsis reproduction, 1 pollen tube exits the transmitting tract at the septum near each ovule and follows the funiculus to the micropylar entrance to the embryo sac (Zhong et al. 2022). This suggests an early polytubey block that prevents more than 1 pollen tube from exiting at a time. This block is released if the sperm cells from the first pollen tube cannot complete double fertilization. Interestingly, 3 members of the CrRLK1L family are expressed in the septum: FER, ANJ, and HERCULES RECEPTOR KINASE1 (HERK1). *fer* single mutants and *herk1/anj* double mutants have a disrupted early polytubey block where multiple pollen tubes emerge from the septum at the same ovule (Zhong et al. 2022). By analogy with other CrRLK1L signaling pathways, Zhong et al. predicted that RALF peptides from the pollen tube might act as ligands for these receptors on the septum. They identified 5 RALF genes with decreased or no expression in the pollen tube *triple myb* mutant: RALFs 6, 7, 16, 36, and 37. Multiple mutant combinations of these RALFs disrupted the septum polytubey block, leading to a model that the first pollen tube that emerges from the transmitting tract near an ovule secretes RALF 6, 7, 16, 36, and 37, which bind to the extracellular domains of FER/ANJ/HERK1 to activate downstream signaling that prevents other pollen tubes from exiting the transmitting tract through an unknown mechanism. The first pollen tube is attracted to the ovules by a gradient of LURE peptides and received by one of the synergid cells, leading to pollen tube rupture and the delivery of the sperm cells (see next sections for more details about this process). If the first pollen tube bursts but the sperm cells do not fertilize the gametes, then the RALFs are no longer produced, the FER/ANJ/HERK1 receptors are inactivated, and the polytubey block at the septum is released so that a new pollen tube can try to reach the ovule. The RALF/FER/ANJ/HERK1 model explains why 1 pollen tube leaves the transmitting tract near each ovule; however, the mechanism that leads to pollen tube exit from the transmitting tract remains a mystery. A signal from the funiculus/septum junction is likely involved in inducing a change in pollen tube growth polarity that turns the pollen tube toward the ovule. The pollen tube must then navigate its way through the septum to emerge near the funiculus.

Synergid cells regulate pollen tube attraction and reception

The pollen tube's path from the stigma through the transmitting tract and the emergence near an ovule's funiculus all require interactions with diploid, sporophytic cells. The final stages of plant reproduction require interactions with the haploid female gametophyte, also known as the embryo sac, which is enclosed in the sporophytic cells of the ovule. In Arabidopsis, the female gametophyte is a highly polarized structure comprised of 7 cells: 2 synergid cells, an egg cell, a diploid central cell, and 3 antipodal cells (Fig. 1). During megasporogenesis, after 3 rounds of mitosis, the syncytium of 8 nuclei differentiates into the 4 cell types (Hater et al. 2020). The first female gametophytic cell type encountered by the pollen tube is the receptive synergid cell. Synergid cells

are highly polarized with a large vacuole at the chalazal end and a membrane-rich region known as the filiform apparatus at the micropylar end (Mansfield et al. 1991; Huang and Russell 1992). The filiform apparatus is located near the entry to the embryo sac and is the site of signaling between the receptive synergid cell and the pollen tube (Mansfield et al. 1991; Leshem et al. 2013). Polarized secretion of signaling molecules during pollen tube attraction and reception might contribute to the accumulation of membrane at the filiform apparatus. Synergid differentiation is regulated by the MYB98 transcription factor (Kasahara et al. 2005). *myb98* mutants have an abnormal filiform apparatus that lacks the extensive invaginations of the wild type and are deficient in pollen tube guidance into the micropyle (Kasahara et al. 2005). Synergid fate is also post-transcriptionally regulated by pre-mRNA PROCESSING factor 8 paralogs (PRP8A and PRP8B) (Kulichová et al. 2020). Expression of MYB98 is reduced in *prp8a/prp8b* double mutants and embryo sac morphology is altered, with some ovules having a collapsed embryo sac or synergid cells that protrude further into the micropyle (Kulichová et al. 2020). Polarity of synergid cells is maintained by longitudinal actin and microtubule networks. When F-actin is disrupted, the formation of the filiform apparatus and location of the central vacuole is altered; when microtubules are disrupted, the invaginations of the filiform apparatus are shorter (Susaki et al. 2023).

Polarized secretion of pollen tube attractants by the synergids

Before pollen tube arrival, the synergid cells are highly polarized secretion machines. Small cysteine-rich peptides called LUREs are secreted from the filiform apparatus and diffuse through the apoplast into the surrounding integument cells (Okuda et al. 2009; Takeuchi and Higashiyama 2012). This secretion is dependent on 1-aminocyclopropane-1-carboxylic acid (ACC) signaling (Mou et al. 2020). In the ACC SYNTHASE (ACS) octuple mutant, LURE1.2 is retained in the synergids and not trafficked to the filiform apparatus and secreted into the micropyle (Mou et al. 2020). ACC increases ovular calcium levels, and treating *acs* mutant ovules with an ionophore rescues LURE retention, suggesting that ACC-induced calcium signaling promotes LURE secretion (Mou et al. 2020). LUREs bind to the pollen tube tip–localized POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 6, which leads to reorientation of the pollen tube and growth toward the micropylar end of the embryo sac (Takeuchi and Higashiyama 2016). LURE1.2 was also identified as the ligand for a receptor heteromer comprised of the leucine-rich repeat kinases MALE DISCOVERER1 (MDS1), MDS2, and MDS1-INTERACTING RECEPTOR-LIKE KINASE 1 (MIK1) and MIK2 (Wang et al. 2016). XIUQIU and TICKET are another group of small cysteine-rich peptide attractants secreted from the filiform apparatus in the Brassicaceae (Meng et al. 2019; Zhong et al. 2019). Unlike LUREs and TICKETs, which promote conspecific pollen tube attraction, XIUQIU are nonspecific attractants (Meng et al. 2019; Zhong et al. 2019). The polarized secretion of both LURE and XIUQIU peptides from the filiform apparatus is reliant on F-actin networks in the synergids before pollen tube reception (Susaki et al. 2023). Remodeling of the actin cytoskeleton in the persistent synergids may be linked to the establishment of synergid polytubey barriers during pollen tube reception by the synergids (Susaki et al. 2023).

Polarity and signaling in pollen tube reception

After a pollen tube is attracted to an ovule, pollen tube reception occurs between the receptive synergid and the pollen tube. Intercellular signaling culminates in pollen tube entry into the embryo sac and bursting to release the sperm cells to achieve double fertilization (Johnson et al. 2019). Synergid proteins involved in pollen tube reception are localized to the filiform apparatus. Pollen tube reception mutants disrupt the signaling process and lead to a "pollen tube overgrowth" phenotype where pollen tube attraction is normal, but the pollen tube does not change its polarized growth pattern to allow bursting, leading to pollen tubes that continue to grow and coil around the receptive synergid (Huck et al. 2003; Rotman et al. 2003). Three members of the CrRLK1L protein family (FER, HERK1, and ANJ) are filiform apparatus localized and are involved in pollen tube reception (Escobar-Restrepo et al. 2007; Galindo-Trigo et al. 2020). FER is chaperoned to the filiform apparatus by the GPI anchored protein LORELEI (LRE) and LRE functions as a co-receptor with FER (Li et al. 2015; Liu et al. 2016). Null *fer*, *lre*, and *herk1/anj* double mutants display pollen tube overgrowth and female sterility (Escobar-Restrepo et al. 2007; Capron et al. 2008; Tsukamoto et al. 2010; Liu et al. 2016; Galindo-Trigo et al. 2020). During pollen tube reception, HERK1 and ANJ function redundantly and likely form a receptor complex with FER and LRE at the filiform apparatus to promote pollen tube entry into the embryo sac and bursting (Galindo-Trigo et al. 2020). The ligands for the FER-HERK1/ANJ-LRE receptor complex are secreted from the arriving pollen tube. The early polytubey block RALFs (6, 7, 16, 36, and 37) also play a role in pollen tube reception; all these RALFs can interact with FER/HERK1/ANJ in in vitro assays and lead to pollen tube overgrowth when mutated (Zhong et al. 2022). In addition, the pollen tube integrity-related RALF4/19 can also interact with the extracellular domain of FER (Gao et al. 2022).

Another protein involved in pollen tube reception is the MILDEW RESISTANCE LOCUS O (MLO) protein NORTIA (NTA, MLO7) (Kessler et al. 2010). In synergids, NTA is Golgi localized before pollen tube arrival in the micropyle but accumulates at the filiform apparatus during pollen tube arrival in a signal-mediated process involving ligand reception by the FER-HERK1/ANJ-LRE complex during pollen tube arrival at the synergids (Kessler et al. 2010; Galindo-Trigo et al. 2020). Even though all members of the MLO gene family are predicted to encode integral membrane

proteins 7 membrane-spanning domains, a range of subcellular localizations have been observed (Jones and Kessler 2017; Jones et al. 2017). When expressed ectopically in synergids, MLO1 accumulates at the filiform apparatus instead of the Golgi, while MLO8 accumulates in trans-Golgi compartments and the filiform apparatus (Jones et al. 2017). The C-terminal, calmodulin-binding cytoplasmic tail of MLO proteins is important for determining subcellular localization (Jones et al. 2017; Ju et al. 2021). A synergid-expressed chimeric protein generated by swapping C-terminal cytoplasmic tail of NTA with that of MLO1 (termed faNTA) is constitutively localized at the filiform apparatus both before and during pollen tube reception (Ju et al. 2021). faNTA is able to bypass FER signaling and suppresses *fer* infertility, suggesting that the presence of NTA at the filiform apparatus of the synergids is sufficient to induce pollen tube bursting independent from FER activity (Ju et al. 2021). These data led to the hypothesis that NTA recruitment to the filiform apparatus downstream of FER activation provides a "booster function" to amplify the FER-mediated synergid response to the arriving pollen tube.

Polarized calcium channels in the synergids regulate pollen tube reception

Pollen tube reception requires communication between the pollen tube and the synergid cells. After following a gradient of synergid-secreted LURE peptides to the female gametophyte, the pollen tube pauses its growth near the filiform apparatus for several minutes before resuming its growth and bursting to release its sperm cargo (Denninger et al. 2014; Ngo et al. 2014). Early hints that calcium may be involved in the communication between the pollen tube and synergids came from live-imaging of calcium reporters in the pollen tube and synergids. Four independent studies concluded that cytoplasmic calcium oscillations initiate in the synergids upon pollen tube arrival in the micropyle (Iwano et al. 2012; Denninger et al. 2014; Hamamura et al. 2014; Ngo et al. 2014). Concurrently, cytoplasmic calcium levels at the pollen tube tip also increase as the pollen tube approaches and communicates with the receptive synergid cell (Iwano et al. 2012; Ngo et al. 2014). In *fer* and *lre* mutants, calcium oscillations are not triggered in the synergids, but in *nta* mutants calcium oscillations occur but are at a lower amplitude (Ngo et al. 2014). These data suggested that FER signaling was necessary to establish the initial calcium oscillations, but NTA acts later to boost the oscillations to the levels necessary for pollen tube bursting and could explain why NTA is sequestered in a Golgi-associated compartment until it is needed at the filiform apparatus to regulate calcium influx during synergid-pollen tube communication (Kessler et al. 2010; Ju et al. 2021). Members of the MLO protein family, including NTA, were recently demonstrated to function as calcium permeable channels (Gao et al. 2022, 2023). This study tested the hypothesis that MLO proteins can act as calcium channels by expressing Arabidopsis, barley, and moss MLOs in mammalian cells and assaying their ability to mediate calcium influx from the surrounding media. Several MLOs, including faNTA (NTA-MLO1$_{cterm}$), could act as calcium channels when expressed alone in animal cells, but NTA could only transport calcium when it was coexpressed with FER and LRE. Interestingly, NTA accumulated in a punctate pattern when expressed alone in mammalian cells, but it was targeted to the plasma membrane if FER and LRE were also expressed. This led the authors to propose that FER and LRE act as coreceptors to recruit NTA to the plasma membrane to form a trio receptor-channel complex. RALF4 and RALF19 peptides can act as FER/LRE ligands to increase FER/LRE/NTA-mediated calcium influx in both mammalian cells and synergids (Gao et al. 2022). Whether RALF6/17/16/36/37, the pollen-expressed RALFs whose multiple mutant led to pollen tube overgrowth (Zhong et al. 2022), can also mediate calcium influx in synergids through NTA is not known. While intriguing, the receptor-channel trio model is based on protein overexpression experiments in heterologous systems. In synergids, LRE has been shown to act as a chaperone for FER, with both proteins accumulating at the filiform apparatus during synergid differentiation and before pollen tube arrival (Li et al. 2015). NTA is not detected at the filiform apparatus before pollen tube arrival (Kessler et al. 2010; Jones and Kessler 2017; Jones et al. 2017; Ju et al. 2021). If LRE (and FER by association) are also chaperones of NTA in synergids, then NTA would need to either be selectively associated with FER/LRE in the Golgi or endocytosed back to the Golgi to prevent early accumulation and calcium channel activity in synergids before pollen tube arrival (Fig. 2, A–C). Alternatively, FER/LRE activation by the RALF peptides could lead to signal transduction that positively regulates NTA trafficking from the Golgi to the filiform apparatus. This would allow for precise control of calcium influx into the synergids after the pollen tube has been recognized (Fig. 2D). Additional studies are needed to better understand the regulation of NTA trafficking in synergids before and during pollen tube reception.

Polarized calcium channels regulate pollen tube tip integrity

A major question in pollen tube reception is "how do the calcium oscillations in the receptive synergid cell relate to the communication with the arriving pollen tube that leads to pollen tube rupture in order to release the sperm cells at the correct time and place?" Recent studies have revealed that similar molecular players are involved in regulating polar growth in the pollen tube. Calcium oscillations also occur in the pollen tube during its journey to deliver the sperm cells to the female gametophyte and are necessary for maintaining polarized tip growth (reviewed in (Zheng et al. 2019). In addition to the ANX1/2-BUPS1/2-LLG2/3-RALF4/9 signaling module discussed earlier in this update, 4 MLO genes are also expressed in pollen tubes (MLO1, MLO5, MLO9, and MLO15) (Meng et al. 2020). Genetic studies revealed a

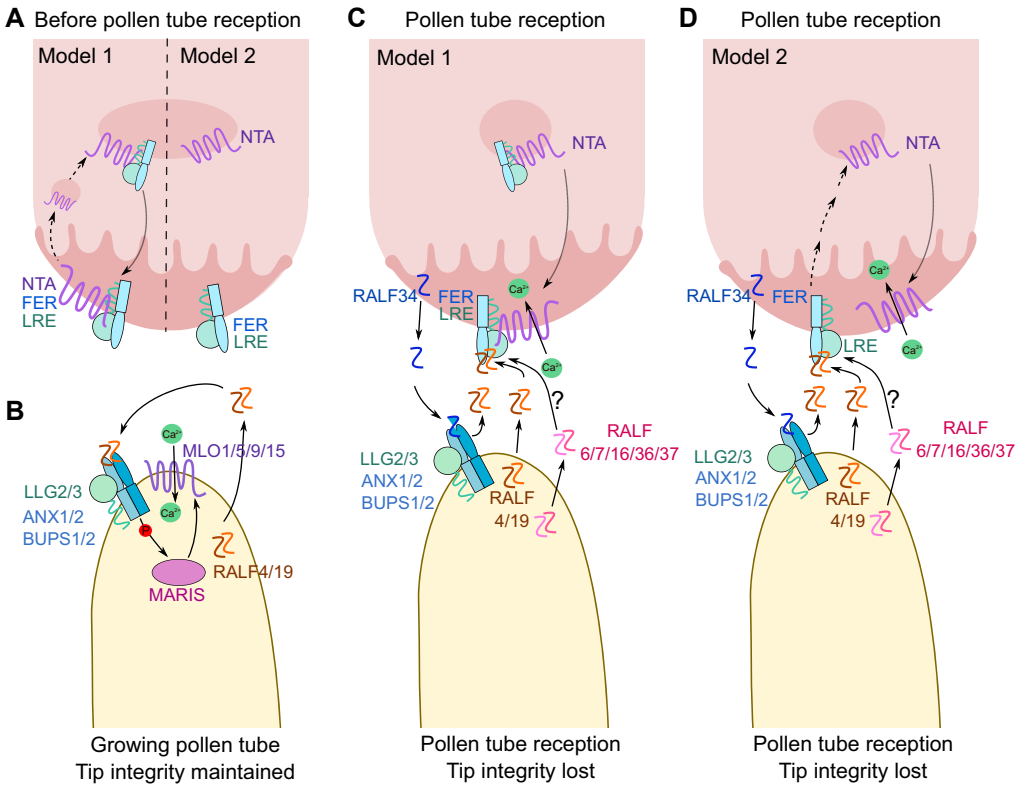

Figure 2. Model of CrRLK1L mediated activation of MLOs before and during pollen tube reception. **A)** Synergids prior to pollen tube arrival. *Model 1:* FER and LRE continuously chaperone NTA from the Golgi to the filiform apparatus where NTA is continuously endocytosed back to the Golgi to keep it inactive. *Model 2*: LRE chaperones FER to the filiform apparatus, while NTA remains Golgi retained. **B)** Pollen tube during active tip growth. RALF4/19 are secreted from the pollen tube and bind to a receptor complex of ANX/BUPS/LLG as a form of autocrine signaling. RALF binding activates MARIS through phosphorylation allowing MARIS to activate MLO1/5/9/15 through an unknown signaling mechanism culminating in Ca^{2+} influx. **C)** *Model 1* during pollen tube reception. RALF4/19 is secreted from the pollen tube and binds to FER/LRE/NTA at the filiform apparatus, which prevents NTA from being endocytosed and promotes Ca^{2+} influx into the synergid. Synergid secreted RALF34 displaces RALF4/19 from ANX/BUPS/LLG likely leading to a loss in pollen tube tip integrity. Pollen tube secreted RALF6/7/16/36/37 can interact with the extracellular domain of FER, however it is unknown whether they also function as regulators of NTA subcellular localization. **D)** *Model 2* during pollen tube reception. RALF4/19 is secreted from the pollen tube and binds to FER/LRE causing a signaling cascade that leads to NTA redistribution from the Golgi to the filiform apparatus. At the filiform apparatus, NTA contributes to Ca^{2+} influx into the synergid. Synergid secreted RALF34 displaces RALF4/19 from ANX/BUPS/LLG likely leading to a loss in pollen tube tip integrity. Pollen tube secreted RALF6/7/16/36/37 have been shown to interact with the extracellular domain of FER, however it is unknown whether they also function as regulators of NTA subcellular localization.

common link between these genes: *anx1/anx2*, *llg2/3*, *ralf4/19*, and *mlo1/5/9* mutant combinations all lead to a failure to maintain pollen tube tip growth leading to premature pollen tube bursting and infertility, suggesting that these molecules may be involved in a common signaling pathway (Boisson-Dernier et al. 2013; Mecchia et al. 2017; Ge et al. 2019b; Gao et al. 2023). In addition to those genes, a cytoplasmic kinase, MARIS (MRI), also plays a role in pollen tube tip integrity. Loss-of-function *mri* pollen tubes burst prematurely, while a point mutation (*mri*^{R240C}) that makes MRI's kinase domain constitutively active is able to complement pollen tube bursting in the *anx1/2* mutant but not in *lrx8-11* mutants that have been linked to RALF4/19 perception (Boisson-Dernier et al. 2015; Franck et al. 2018). As was seen with the FER/LRE/NTA signaling module in synergids, all of these loss-of-function pollen tube mutants had reduced cytoplasmic calcium levels at the pollen tube tip. Exogenously applied RALF4/19 increased calcium uptake into the pollen tube (Gao et al. 2023), but whether this occurs with endogenous levels of RALF peptides during in vivo pollen tube growth through the pistil remains to be determined. In addition, the constitutively active *mri*^{R240C} mutant had increased calcium levels in hydrating pollen grains (Gao et al. 2023). Taken together, these data revealed a similar signaling pathway to that seen in receptive synergids, with RALF4/19 peptides activating an RLK pathway that upregulates calcium influx through MLO channels (Fig. 2B). In contrast to the synergid system, where NTA coexpressed with FER and LRE in mammalian cells caused calcium influx, pollen tube–expressed MLO proteins could only function as calcium channels in mammalian cells if they were expressed in combination with ANX1/2-BUPS1/2-LLG2/3-MRI or with MRI^{R240C} alone. This led to the hypothesis that RALF4/19 interaction with the ANX1/2-BUPS1/2-LLG2/3 coreceptor

triggers phosphorylation of MRI, which then activates the MLO channels. In vitro phosphorylation tests showed that BUPS1 can phosphorylate MRI, but MRI does not phosphorylate the MLOs tested (Gao et al. 2023). Thus, the mechanism through which MRI^{R240C} activates MLO calcium channels in mammalian cells remains a mystery.

The pollen-expressed MLO proteins may also function in regulating pollen tube growth direction and speed (Meng et al. 2020). *mlo5/9* and *mlo5/9/15* mutants are able to maintain tip growth but grow more slowly and make extra turns on their way to the ovule, resulting in coiled pollen tubes that pile up outside of ovules. Consistent with a role for MLOs as calcium channels, these mutant pollen tubes had decreased Ca^{2+} in their tips. The milder mutant phenotype allowed Meng et al. to investigate other aspects of MLO function in pollen tube and conclude that MLO5/9 have polar accumulation associated with changes in growth direction and that another calcium channel, CNGC18, is also involved in polarized Ca^{2+} influxes associated with pollen tube guidance. Whether the guidance function of MLO5/9/15 is related to RALF/ANX/BUPS/LLG signaling remains to be determined.

Bringing it all together at pollen tube reception

The synergid and pollen tube signaling systems during pollen tube reception both rely on RLK activation by RALF4/19 peptides that are expressed and secreted at the pollen tube tip (Fig. 2). A third RALF peptide, RALF34, is secreted from synergids and was proposed to displace RALF4/19 from the ANX1/2-BUPS1/2 receptor complex at the pollen tube tip to promote pollen tube bursting during pollen tube reception (Ge et al. 2017). The authors of this study proposed a model where autocrine signaling mediated by RALF4/19 promotes pollen tube growth during its journey to the ovule, while ligand exchange with RALF34 secreted from the synergids occurs during pollen tube reception to disrupt the pollen tube integrity mechanism by paracrine signaling (Ge et al. 2017). The finding that 5 additional pollen tube RALFs (6, 7, 16, 36, 37) are necessary for pollen tube bursting (Zhong et al. 2022) complicates this model; perhaps these RALFs are necessary for triggering FER/LRE-mediated release of RALF34. The recent studies implicating RALF4/19 in MLO-mediated calcium influx into both the growing pollen tube and the synergids during pollen tube reception (Gao et al. 2022, 2023) suggest a possible additional layer of paracrine signaling, where the RALF4/19 peptides that are displaced from the pollen tube tip could interact with the FER/LRE receptor in the filiform apparatus to stimulate calcium oscillations in the synergids (Fig. 2, C and D). This model predicts that RALF34 interaction with the ANX/BUPS receptor complex in the pollen tube tip might interfere with calcium influx. However, live imaging of calcium dynamics in the pollen tube as it communicates with the synergids before resuming its growth and bursting showed that calcium levels actually increase in the tip of the pollen tube during this timeframe (Ngo et al. 2014). This suggests that RALF34 may play some other role in regulating pollen tube tip integrity and bursting during reception at the synergid. A second prediction is that if both ANX/BUPS and FER extracellular domains interact with RALF4/19, then the ANX extracellular domain may be able to substitute for the FER extracellular domain in synergids. Domain swaps between FER and ANX1 revealed that the intracellular domain of ANX1 could substitute for the FER domain and complement the *fer-1* pollen tube reception phenotype, but the ANX1 extracellular domain could not (Kessler et al. 2015). However, it is possible that other pollen tube–specific components from the ANX/BUPS/LLG receptor complex are necessary for the ANX1 extracellular domain to be able to interact with RALF4/19.

While it is clear that maintenance of a proper calcium gradient is essential for pollen tube tip growth and that disruption of calcium transport would interfere with tip integrity (Scheible and McCubbin 2019), it is less clear why synergids have increased calcium uptake and oscillations during communication with the pollen tube during reception. One possibility is that synergid calcium oscillations are related to synergid degeneration during pollen tube reception, and this degeneration is necessary for inducing pollen tube rupture. A second possibility is that the calcium oscillations in the receptive synergid play a role in making certain that the pollen tube tip bursts in close proximity to the egg and central cell nuclei to ensure that the sperm cells have the best chance at double fertilization. Live imaging experiments revealed that the pollen tube probably does not penetrate the receptive synergid after pausing for several minutes to an hour at the filiform apparatus and resuming its growth shortly before bursting (Ngo et al. 2014; Ju et al. 2021). Instead, the pollen tube appears to grow along the surface of the receptive synergid. Perhaps calcium signaling causes changes in synergid cell biology that encourage the pollen tube to grow in contact with the synergid plasma membrane in its approach to the egg cell. These changes could involve protein trafficking or phospholipid signaling, 2 processes that have been linked to calcium signaling (reviewed in Himschoot et al. 2017). A third possibility is that calcium transport into the synergids by NTA at the filiform apparatus acts to deplete calcium in the filiform apparatus and micropylar space, which changes the extracellular environment experienced by the pollen tube tip and leads to remodeling of the cell wall to promote tip rupture as the pollen tube resumes growth toward the egg.

Turning off the synergid signals

After pollen tube reception, a polytubey block is established to prevent multiple pollen tubes from being attracted to and fertilizing the same ovule. This polytubey block occurs as the pollen tube arrives at the ovule and nitric oxide accumulates in a FER-dependent signaling pathway (Duan et al. 2020). The

Box 2.

Large gene families encode reproduction signaling components. During reproduction, CrRLK1Ls and LRE/LLGs act as co-receptors for RALF peptide ligands in a signaling process that activates MLOs in the pollen tube, synergid, and potentially throughout the pistil (Table 1). These signaling molecules are encoded by large gene families in Arabidopsis, indicating possible functional divergence over the course of plant evolution. The CrRLK1Ls are a family of 17 plasma membrane localized receptor-like kinases that have extracellular domains with variable numbers of malectin binding domains and a cytosolic kinase domain (Boisson-Dernier et al. 2011). The RALF protein family consists of 37 members that function as secreted cysteine-rich peptide ligands (Abarca et al. 2021). The 15 members of the MLO gene family are seven-transmembrane domain proteins with C-terminus calmodulin binding domains (Devoto et al. 2003). So far, 10/15 of the Arabidopsis MLO proteins have been shown to have Ca^{2+} channel activity in plantae or when expressed in mammalian cells (Gao et al. 2022, 2023). Finally, there are 4 LRE/LLG GPI-anchored proteins believed to chaperone FER to the plasma membrane and act as co-receptors with FER or other members of the CrRLK1L family (Noble et al. 2022). Despite being from large protein families, the same CrRLK1L and RALF family members are often reused throughout different stages of pollen-pistil interactions (Table 1), but LRE/LLG and MLO family members tend to be differentially expressed in reproductive tissues. In sporophytic tissues, FER also interacts with LLGs to perceive RALF ligands during development and in response to biotic and abiotic stress (Zhu et al. 2021). While many of the CrRLK1L and MLO family members remain uncharacterized, it is possible that various combinations of these proteins form signaling modules that are repeated throughout the plant.

Box 2: Table 1. Signaling proteins involved in different stages of plant reproduction

Stage	Tissue	CrRLK1L	LRE/LLG	RALF	MLO	References
Pollen hydration and germination	Stigma	FER ANJ	LLG1	RALF33	?	Liu et al. (2021)
	Pollen	ANX1/2 BUPS1/2	LLG2/3	RALF4/19	?	Ge et al. (2017, 2019b), Gao et al. (2023)
Tip growth through reproductive tract	Pollen tube	ANX1/2 BUPS1/2	LLG2/3	RALF4/19	MLO1/5/9/15	Boisson-Dernier et al. (2009), Miyazaki et al. (2009), Ge et al. (2017, 2019b), Mecchia et al. (2017), Zhu et al. (2018), Meng et al. (2020), Zhou et al. (2021), Gao et al. (2023)
Early polytubey block	Septum	FER ANJ HERK1	?	?	?	Zhong et al. (2022)
	Pollen tube	?	?	RALF6/7/16/36/37	?	Zhong et al. (2022)
Pollen tube reception	Synergid	FER ANJ HERK1	LRE	RALF34	NTA (MLO7)	Huck et al. (2003), Rotman et al. (2003), Escobar-Restrepo et al. (2007), Capron et al. (2008), Kessler et al. (2010), Liu et al. (2016), Ge et al. (2017), Galindo-Trigo et al. (2020), Ju et al. (2021), Gao et al. (2022)
	Pollen tube	ANX1/2 BUPS1/2	LLG2/3	RALF4/19/6/7/16/36/37	?	Miyazaki et al. (2009), Boisson-Dernier et al. (2013), Ge et al. (2017, 2019b), Zhu et al. (2018), Gao et al. (2023)

nitric oxide nitrosates LUREs, which was proposed to decrease LURE efficacy as chemoattractants and inhibits their secretion (Duan et al. 2020). After the egg cell is fertilized, aspartic endopeptidases EGG CELL SPECIFIC 1 (ECS1) and ECS2 are secreted, which cleave LURE peptides to prevent polytubey (Yu et al. 2021). Pollen tube arrival in the micropyle promotes the degeneration of the receptive synergid but not the persistent synergid (Sandaklie-Nikolova et al. 2007; Leydon et al. 2015). The persistent synergid remains intact until fertilization occurs so an additional pollen tube can be attracted should fertilization fail. Death of the persistent synergid is induced after fertilization, culminating in fusion with the fertilized central cell (Maruyama et al. 2015). The arabinogalactan protein AGP4 (also known as JAGGER) is necessary for persistent synergid degeneration (Pereira et al. 2016). Ethylene signaling components have also been implicated in synergid-central cell fusion (Völz et al. 2013; Maruyama et al. 2015). However, ethylene itself does not seem to be involved in synergid death and fusion, indicating a more complex mechanism involving crosstalk with ethylene-related transcription factors (Li et al. 2022).

The final frontier: double fertilization

Plant reproduction is completed when the 2 sperm cells fertilize the egg and central cells. The egg cell is highly polarized

OUTSTANDING QUESTIONS BOX

- The CrRLK1L/LLG/RALF signaling module is repeated at several stages during plant reproduction. What are the downstream cellular events that lead to the different outcomes (pollen hydration, polytubey block at the septum, pollen tube tip integrity vs bursting to release the sperm cells)?
- How does the Ca^{2+} influx mediated by MLO activation by the CrRLK1L/LLG/RALF signaling module relate to ROS production that is regulated by CrRLK1L signaling?
- How are the MLO-mediated Ca^{2+} oscillations in the synergids during pollen tube reception related to regulating pollen tube tip growth to induce bursting?
- The CrRLK1L/LLG/RALF/MLO gene families are found throughout the plant kingdom. Is the signaling module conserved in all flowering plants and in other plant lineages?

with the organelles and nucleus at the chalazal end and a large micropylar vacuole (Mansfield et al. 1991). In contrast to the preceding steps of reproduction where polarized RLKs interact with ligands to control pollen tube behavior, so far no *CrRLK1L*, *LLG*, *RALF*, or *MLO* genes have been implicated in gamete interactions in Arabidopsis. This could reflect a more ancient origin of the gametes compared with the pollen tube (Sharma et al. 2021). However, similar to pollen tube–synergid interactions, egg–sperm interactions involve signal-triggered polarized protein secretion and Ca^{2+} changes. GAMETE EXPRESSED 2 is a sperm-expressed plasma membrane protein of unknown function required for gamete adhesion (Mori et al. 2014). After adhesion of the sperm cell to the egg or central cell, gamete fusion occurs to complete double fertilization. HAPLESS 2, also known as GENERATIVE CELL SPECIFIC 1 (HAP2/GCS1), is a sperm cell–expressed fusogenic membrane protein required for gamete fusion (Mori et al. 2006; von Besser et al. 2006; Liu et al. 2008). Sperm cell activation is required to separate the sperm cell pair and activate the proteins required for membrane fusion. Polar secretion of EGG CELL 1 (EC1) peptides from the egg cell contributes to sperm cell activation by inducing the redistribution of sperm HAP2/GCS1 from internal compartments to the plasma membrane, where it is functional (Sprunck et al. 2012). The EC1-induced polar redistribution of HAP2/GCS1 is dependent on 2 DOMAIN OF UNKNOWN FUNCTION 679 proteins (DMP8 and DMP9) (Wang et al. 2022). Upon successful egg–sperm fusion, a short calcium transient is observed that is not generated upon central cell fusion (Denninger et al. 2014). The Ca^{2+} channels leading to and the signaling pathways activated by this calcium transient have not yet been identified.

Conclusions

In the past 3 years, significant advances have been made in understanding RLK-ligand control of pollen tube behavior during pollen-pistil interactions. The RALF/CrRLK1L/LLG/MLO signaling modules described in this update all involve members of large gene families (Box 2). This signaling module is not restricted to plant reproduction. Instead it appears to have been co-opted for a variety of signaling processes within the plant. Various members of these gene families have been implicated in root development, plant–pathogen interactions, plant stress, and hormone responses, etc. (Kusch and Panstruga 2017; Zhu et al. 2021; Noble et al. 2022). The next challenge will be to determine how these signaling pathways lead to such diverse outcomes during plant reproduction, growth and development, and responses to the environment (see "Outstanding Questions").

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S.T.O. and S.A.K. conceived, wrote, and edited the article.

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