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Extracellular Guidance Cues and Intracellular Signaling Pathways that Direct Pollen Tube Growth

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Abstract Fertilization in flowering plants requires that a pollen tube deliver two sperm to the female gametes, which develop in ovules buried deep within floral tissues. The tube germinates on a receptive stigma and enters the style where it grows rapidly in a nutrient-rich extracellular matrix secreted by cells of the transmitting tract (Lord 2003). Subsequently, it enters the ovary where it continues to grow on the surface of cells while targeting an individual ovule. Inside the ovule, the pollen tube immediately encounters the haploid synergid cells and continues to grow through the filiform apparatus, a specialized cell wall that forms at the basal junction of the two synergids. The journey ends when the tip enters one of the two synergids and bursts.

How does the pollen tube navigate these diverse environments within the pistil to reach a precise cellular target? Recently a great deal of progress has been made toward defining the sources of signals that direct specific stages of the pollen tube journey and toward identifying molecules that direct tube growth. However, our understanding of how the tube changes direction of growth in response to signals presented by floral cells along its path is still limited. For example, no pollen tube receptors have been identified for any of the extracellular guidance cues identified thus far and consequently, it has not been possible to assign specific signal transduction pathways linking the floral environment to changes within the pollen tube that cause reorientation of the tip. Here we review the recent progress toward identification of extracellular guidance cues and highlight efforts to understand how the tube perceives and transduces these signals into changes in the direction of its growth.

1 Introduction

The male gametophyte, or pollen grain, develops in the anther from a microspore and consists of a vegetative cell that contains two sperm cells (Twell et al., this volume). The nucleus of the vegetative cell (or tube cell) and the two sperm, which together make up the male germ unit, migrate near the tip of the pollen tube as it extends toward an ovule (Twell et al., this volume). The

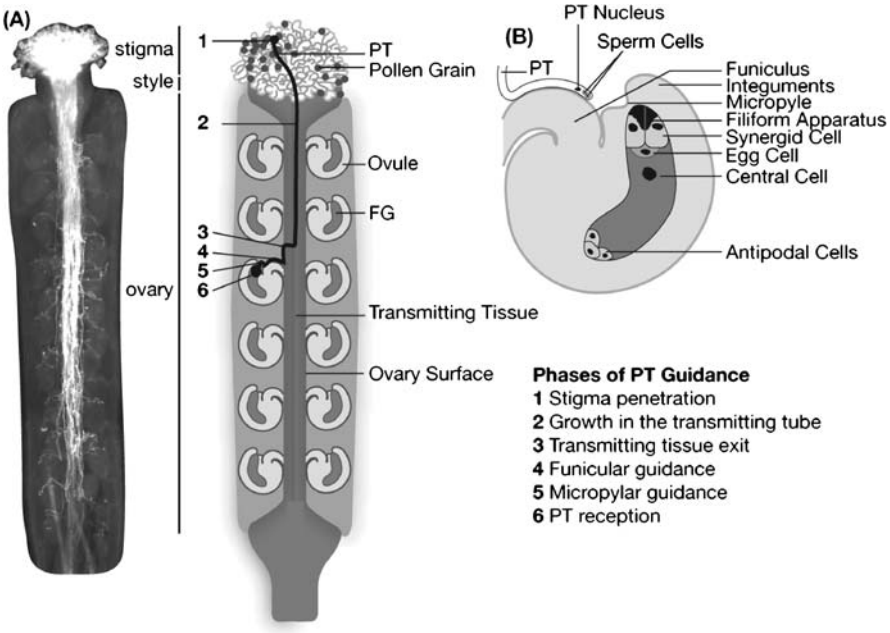

Fig. 1 Phases of pollen tube guidance. **A** The tube path is shown in an aniline blue stained Arabidopsis pistil (*left*) and in a schematic (*right*). **B** Critical cells and structures of the male and female gametophytes

female gametophyte, or embryo sac, develops within an ovule from a megaspore and most commonly consists of seven-cells (Yadegari and Drews 2004). The egg and the two synergids develop at the micropylar pole, three antipodal cells are located at the chalazal pole, the central cell is the largest cell and lies between the other two groups of cells. With the exception of the central cell, which is produced by fusion of a cell from each pole, each cell is haploid (Yadegari and Drews 2004).

Pollen tubes only encounter sporophytic cells on their way to the ovule and do not interact with gametophytic cells until they stop growing and burst within one of the synergids. However, there is growing evidence that pollen tube guidance is regulated by collaboration between sporophytic and gametophytic cells of the female tissue.

2 Major Models for Pollen Tube Guidance: Floral Architecture and Chemotropism

Two major hypotheses have been proposed to explain the precise growth of pollen tubes to ovules (Heslop-Harrison 1986, 1987). One holds that pistil

architecture dictates the path and that the pollen tube simply follows a mechanical trail of least resistance to the micropyle. Support for this model comes from anatomical studies of many species showing that the transmitting tissue of the style provides a defined environment for pollen tube growth that leads directly to the ovary. In the ovary, epidermal cells are arranged in files that lead to ovules and pollen tubes have been observed to grow between these files of cells (Shimizu and Okada 2000). In this hypothesis, the ECM of cells along the path would play a major role in guidance providing adhesive molecules that would keep the pollen tube on the prescribed path (Lord 2003), or in some cases may drive tube migration by pulling it along its path through a matrix-driven adhesion mechanism (Sanders and Lord 1989).

The chemotropic hypothesis posits that pollen tubes are directed to their target or along a series of intermediate targets by molecular guidance cues that change the direction of tube elongation. In this model, a target cell or cells produce molecules that either attract or repel pollen tubes. These molecules could be present in a gradient that continuously focuses the direction of the tube tip toward the highest concentration at its source. Such long-standing gradients were discussed to be difficult, if not impossible to attain (Lush 1999). Alternatively guidance cues may be expressed at a location that simply re-directs the tube tip, thereby changing the path of growth; in this case a continuous gradient may not be required and a point-source of the guidance cue may be sufficient (Mascarenhas 1975).

There has been a long history of debate over which of these two models best explain pollen tube guidance and at times the chemotropic model has been vigorously challenged (Heslop-Harrison 1986; Lush 1999). It is now clear from genetic analysis of tube guidance in *Arabidopsis* and in vitro guidance experiments in *Torenia fournieri*, that chemotropism plays a critical role in the final stages of tube growth and it is very likely that flowering plants use a combination of architectural constraints, ECM-derived adhesion molecules, and highly regulated production of chemotropic guidance cues to direct the pollen tube to the ovule.

3
Pollen Tube Guidance Comprises Multiple Steps and Requires Multiple Signals

A series of genetic experiments in *Arabidopsis* showed that tube guidance is a multiphase process in which early stages are controlled by sporophytic cells of the stigma, style, and transmitting tissue and the final stages are controlled by the female gametophyte. These experiments also provide critical support for a chemotropic component for tube guidance because some of the mutants analyzed do not disrupt floral architecture but disrupt tube guidance. Pollen

tube growth in *Arabidopsis* has been divided into the following phases (Huck et al. 2003; Johnson and Preuss 2002; Kandasamy et al. 1994; Rotman et al. 2003): (1) Stigma penetration, (2) Growth in the style and transmitting tissue, (3) Emergence from the transmitting tissue, (4) Funicular guidance, (5) Micropylar guidance and (6) Pollen reception.

Pollen tubes growing in pistils with homozygous mutations that disrupt ovule and FG development follow a chaotic path once they emerge from the transmitting tract and begin to grow on the ovary surface; tubes do not grow toward a funiculus, but grow on other surfaces including the ovary wall (Hülskamp et al. 1995). However, early stages of tube growth such as penetration of the stigma and rapid polar extension through the transmitting tissue of the style were not affected by these mutations. These experiments showed that tube guidance in the ovary is controlled by a mechanism distinct from that in the stigma and style and that the ovule and/or FG control pollen tube guidance in the ovary. Interestingly, the distribution of pollen tube exit points from the transmitting tract was also altered in these mutants, suggesting that ovule and/or the FG controls the position of tube exit from the transmitting tract (Hülskamp et al. 1995).

To determine whether sporophytic cells of the ovule or haploid FG cells direct pollen tube guidance following emergence from the transmitting tract, mutants were examined that block inception of FG development without affecting ovule development (Ray et al. 1997). In these mutants, all sporophytic diploid cells were normal, but half of ovules contained no FG. The path of wild-type pollen tubes growing in these mutant pistils was normal except they did not grow toward the funiculi of ovules that contained an aborted FG. This experiment showed that a functional FG was required for pollen tubes to target ovules.

Analysis of the *maa* mutants, which initiate but do not complete FG development, showed that growth of the pollen tube from the surface of the ovary to the micropyle can be divided into two phases (4 and 5) controlled by distinct mechanisms (Shimizu and Okada 2000). Unlike mutants that block initiation of FG development, *maa* mutants are able to attract pollen tubes to the distal regions of the funiculus, but these tubes fail to make the final abrupt turn required to enter the micropyle (Shimizu and Okada 2000). Therefore, micropylar guidance is controlled by a factor produced by FGs that are mature or near mature, while funicular guidance is controlled by a factor produced earlier in FG development.

In addition, these experiments showed that the FG could control repulsion of supernumerary pollen tubes. Ovules containing *maa* FGs attract multiple pollen tubes that remain stuck on the funiculus, whereas wild-type ovules attract a single pollen tube that grows up the funiculus and enters the micropyle. These results suggest either that the attractive signal is immediately quenched following tube entry into the micropyle, or that a second, repulsive signal is produced immediately following entrance (Shimizu and Okada 2000).

The final phase of guidance occurs when the tube stops growing and bursts. This stage is called pollen tube perception and was defined by analysis of FG-specific mutations called *sirene* and *feronia* (Huck et al. 2003; Rotman et al. 2003). These mutants attract pollen tubes into the micropyle, but these tubes do not burst, instead they continue to grow within the FG forming coils. Interestingly, *feronia* mutants attracted multiple tubes suggesting that entry of a tube into the micropyle is not sufficient to quench the attractive signal or initiate production of a repellant (Huck et al. 2003). These experiments clearly define a role for the FG in directing the final phases (4–6) of tube growth. Do the sporophytic cells of the ovule play any role in these phases? *ino* mutant ovules produce normal FGs, but fail to form the outer integument (Baker et al. 1997). The inner and outer integuments are layers of cells that encase the ovule; the micropyle is formed where these layers of cells converge. Interestingly, tube growth in pistils homozygous for *ino* is chaotic after emergence from the transmitting tract and is reminiscent of tube growth near ovules that completely lack an FG. This suggests that in addition to the FG, the outer integuments may be required for the funicular guidance step. It should be noted that the *ino* mutation also causes the micropyle to be oriented toward the base of the pistil rather than toward the stigma as in wild type. However, *ino* also causes the micropyle to be oriented toward the base of the pistil rather than toward the stigma as in wild type and this dramatic change in ovarian architecture could also account for the tube guidance defect observed in *ino*.

4
The Synergids are the Source of a Chemotropic Factor Directing Micropylar Guidance

The synergids appear to be specialized secretory cells (Higashiyama and Inatsugi, this volume). This feature led to the hypothesis that the synergids were a source for a chemotropic factor directing the pollen tube into the micropyle. This was directly tested using a semi-in vitro system developed for *Torenia fournieri* (Higashiyama et al. 1998; Higashiyama and Inatsugi, this volume) in which tubes grow through an excised style and out on to the surface of a culture medium before targeting the ovules placed on the surface of the medium. The FG of *Torenia fournieri* protrudes from the micropyle facilitating experimental manipulation and making it possible to analyze the role of the FG in tube guidance without intervening sporophytic cells. Laser ablation studies showed that at least one intact synergid was required for pollen tubes to target the FG (Higashiyama et al. 2001). The synergids were found to be the only FG cell type that is essential for attraction; ablation of the egg, central cell, or antipodals had no effect (Higashiyama et al. 2001).

Which phase of tube guidance as defined above for *Arabidopsis* is being analyzed in the *Torenia* system? In the *Torenia* in vitro system, tubes

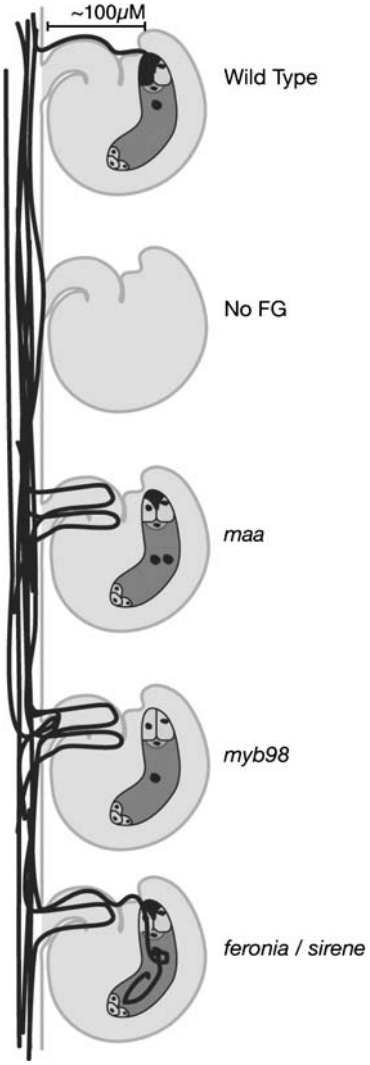

Fig. 2 Arabidopsis FG mutants define phases 3–6 of pollen tube guidance. Wild type ovules attract a single tube that enters the micropyle and bursts within one of the two synergids. Ovules that contain no FG do not attract pollen tubes to the micropyle and also show defects in the patterns of tube exit from the transmitting tissue. Maa mutants attract multiple tubes but these fail to enter the micropyle. Myb98 mutant attracts multiple tubes to the funiculus but fails to attract them to the micropyle. *Feronia* and *sirene* attract tubes that fail to burst within the synergids

grow directly to the filiform apparatus, a specialized area of cell wall at the basal junction of the two synergids; they do not contact sporophytic cells. In vivo, *Torenia* pollen tubes grow directly from the placental surface to

the filiform apparatus without contacting the funiculus (Higashiyama et al. 1998). Thus, *Torenia* appears not to have a phase analogous to funicular guidance in *Arabidopsis*. It is likely that the phase of guidance being interrogated in the *Torenia* system is analogous to the micropylar guidance phase in *Arabidopsis*.

Support for this idea comes from the recent analysis of the Arabidopsis FG mutant, *myb98* (Kasahara et al. 2005). All cells of *myb98* FGs develop normally except the synergids, which have a subtle and specific defect in the ultrastructure of the filiform apparatus. MYB98 is expressed specifically in the synergid cells and possibly functions as a transcription factor that regulates development of the filiform apparatus. *Myb98* FGs attract pollen tubes to the funiculus, but have defects in micropylar targeting. These data strongly suggest that the synergids are the source of the micropylar guidance cue in *Arabidopsis* and that the filiform apparatus is required for production and/or secretion of this chemotropic guidance cue (Kasahara et al. 2005). Biochemical purification of the active factor in *Torenia*, further analysis of *Arabidopsis* FG mutants, and analysis of genes that are specifically expressed in the synergids are promising approaches that could lead to identification of this molecule in the near future. Large-scale genetic analysis of the *Arabidopsis* FG has yielded a very interesting group of 18 *une* mutants that have apparently normal FG development but remain unfertilized (Pagnussat et al. 2005). Six *une* mutants were identified that failed to attract pollen tubes; further analysis of the genes disrupted in these mutants, which include genes of unknown function (UNE1, UNE4), a calcium binding protein (UNE14) and a small LEA protein (UNE15) will be an exciting area for future studies. Additionally, analysis of FG-specific gene expression has lead to the identification of a candidate micropylar guidance factor in maize, EA1 (Marton et al. 2005; Sect. 5.4).

5
Chemotropic Molecules Directing Tube Guidance

Chemotropic guidance molecules may be expected to meet the following basic set of experimental criteria: 1) the molecule should be present in a gradient with highest concentration at the target site 2) the molecule should have chemotropic activity (attractive or repellent) in an in vitro assay, 3) manipulation of the factor in vivo should have consequences for the direction of tube growth. Recently, a series of molecules have been postulated to function as chemotropic guidance molecules. Many of these have only been reported in the past few years and none of them yet fulfill all of these experimental criteria for a chemotropic guidance molecule. However, they all have features that are highly suggestive of chemotropic activity.

5.1 Chemocyanin

Lily pollen tubes gain access to the style through pores on the surface of the stigma and it was proposed that a chemotropic factor expressed on the stigma guides tubes that germinate on the surface of the stigma to these pores (Kim et al. 2004). A lily stigma protein preparation was shown to reorient tube growth in vitro. This preparation was further separated into multiple fractions by reverse-phase HPLC and only one of the fractions retained chemotropic activity. Analysis of this fraction showed that it contained one prominent protein with homology to a family of small basic proteins called plantacyanins and was called chemocyanin. Whether chemocyanin expression is focused around pores that lead to the hollow stylar canal has not been addressed but in vitro experiments suggest that chemocyanin functions by attracting tubes up a concentration gradient. Interestingly, chemocyanin activity in vitro was enhanced by inclusion of SCA (Kim et al. 2003), the small cysteine-rich adhesin that had already been shown to function in a complex with pectin to mediate adhesion of pollen tubes to a growth substrate in vitro (Mollet et al. 2000).

Plantacyanins have been found in many plants including *Arabidopsis* where the homolog is 52% identical to lily chemocyanin. Remarkably, recombinant *Arabidopsis* plantacyanin can reorient lily pollen tubes in vitro (Dong et al. 2005). A T-DNA insertion was identified in the *Arabidopsis* plantacyanin gene that reduced, but did not eliminate expression of plantacyanin. These mutants were fully fertile and any subtle defects in tube guidance were not reported. However, plantacyanin overexpression using the strong and ubiquitous cauliflower mosaic virus 35S promoter led to reduced fertility. When pistils overexpressing plantacyanin were pollinated with wild-type pollen, seed set was reduced by about 50% and some tubes were observed to coil around the stigma papillae and grow away from the style rather than toward it. However, many tubes were still able to penetrate the stigma, suggesting other defects within the ovary account for reduced seed set. In *Arabidopsis*, plantacyanin is prominently expressed along the path of the pollen tube and is particularly high in the transmitting tissue, suggesting it may function as a guidance molecule at a later stage of pollen tube growth. Plantacyanin is also expressed in the FG and it will be interesting to determine if plantacyanin plays a role at the terminal phases of tube growth as well.

Chemocyanin has chemotropic function in vitro and experiments in *Arabidopsis* show that manipulating plantacyanins in vivo can have consequences for tube guidance. Whether a gradient of chemocyanin forms near stigmatic pores in lily has not yet been tested and the biochemical function of plantacyanins in tube guidance remains to be elucidated. It will be interesting to determine whether the pollen tube expresses a receptor for plantacyanins.

5.2 GABA

The *pop2* mutant of *Arabidopsis* specifically disrupts pollen tube guidance. However, unlike the *Arabidopsis* mutants described above that define the phases of tube growth, it does not affect any female reproductive structures (Wilhelmi and Preuss 1996). Therefore, this mutation might directly affect the tube guidance system. When *pop2* pollen tubes grow in a *pop2-/-* pistil many of the tubes remain within the transmitting tract and those that exit the transmitting tract generally fail to enter the micropyle (Palanivelu et al. 2003; Wilhelmi and Preuss 1996). Therefore, this mutation disrupts phases 2–5 of tube guidance.

Pop2 mutant pistils accumulate ~ 100 fold more γ-amino butyric acid (GABA) than wild type because of a mutation in a transaminase that normally metabolizes GABA (Palanivelu et al. 2003). GABA is a four carbon ω-amino acid that has been shown to function in cell-cell signaling in many organisms and is best known as an inhibitory neurotransmitter in the human nervous system (Pinal and Tobin 1998). Immunofluorescence studies show that GABA is present in the *Arabidopsis* pistil along the tube growth path with peak concentration in the outer integument cells that surround the micropyle. This led to the proposal that GABA forms a chemotropic gradient that directs the tube to the micropyle. In *pop2* mutants, the abundance of GABA was higher throughout the pistil, and it was suggested that the GABA gradient directing tube growth to the micropyle is disturbed. *Pop2* is unique among guidance mutants described thus far because the mutant phenotype is only observed when the plant is self-fertilized. Wild-type pollen tubes navigate the *pop2* pistil without difficulty; perhaps this is because POP2 tubes retain GABA metabolic activity. Manipulation of GABA levels in vivo has dramatic consequences for tube guidance but chemotropic activity in vitro has not been demonstrated (Palanivelu et al. 2003). There are no obvious homologs of animal GABA receptors in the *Arabidopsis* genome (Palanivelu et al. 2003), so the molecular processes that mediate GABA signaling during pollen tube guidance remain unclear and novel approaches will need to be taken to identify and characterize GABA signaling components critical for pollen tube guidance in *Arabidopsis*.

5.3 TTS Proteins

Arabinogalactan proteins (AGPs) are encoded by a large gene family and display a diverse array of glycosylation patterns; importantly, they are among the most abundant proteins in the ECM of the transmitting tissue (Cheung et al. 1995; Wu et al. 1995). Pollen tubes incorporate AGPs into their cell walls as they extend and a synthetic molecule that binds AGPs has been shown to

disrupt tube growth in vitro (Mollet et al. 2002; Wu et al. 1995). These characteristics all suggest an important role for AGPs in regulating tube growth in the transmitting tissue. Transmitting tissue specific (TTS) AGPs from tobacco were shown to be more heavily glycosylated at the ovary end of the style and less so at the stigma end (Wu et al. 1995). When purified TTSs were placed in pollen tube growth medium they attracted tubes that had emerged from an excised tobacco style (Cheung et al. 1995). Attraction was mild and it is possible that tubes appear to grow toward TTS because they provide sugar and therefore stimulate growth. Transgenic tobacco plants expressing antisense versions of the TTS gene had reduced seed yield that resulted from reduced tube growth through the style; defects in the direction of tube growth were not observed. This suggests that TTS proteins are critical for tube growth in the style, but they may not be chemotropic guidance cues *per se*. TTSs may be required instead for rapid, highly polar growth in the transmitting tract that takes tubes to the ovary. It is thus possible that in the transmitting tissue tubes are not directed by chemotropic factors, but instead are just extending in a straight path that is defined by architectural features of the pistil and supported by nutritional factors such as TTS glycoproteins.

5.4 EA1

Maize *EA1* is expressed only by synergid cells and the egg and encodes a small protein of 94 amino acids with a predicted transmembrane domain (Marton et al. 2005). When EA1 was fused to GFP and expressed from its own promoter in transgenic maize plants, fluorescence was observed in the synergid cell walls of immature FGs. As ovules matured, fluorescence became more intense and spread into the six layers of nucellar cells that form the micropyle in the maize ovule. The *EA1* promoter alone drove reporter gene expression only in the egg and synergids,; thus, accumulation of EA1:GFP in the micropyle must be the result of secretion. Interestingly, EA1:GFP fluorescence did not appear to accumulate evenly in the cells that surround the egg and synergid. Instead EA1:GFP accumulated preferentially toward the micropyle leading to the suggestion that EA1 secretion and accumulation is tightly spatially regulated. This expression pattern is consistent with formation of an EA1 gradient around the micropyle.

Transgenic maize plants expressing either an *EA1* RNAi construct or an *EA1* antisense construct from the ubiquitin promoter had reduced seed set following self-pollination or when transgenic plants were used as the female in crosses with wild type (Marton et al. 2005). This effect was observed in a subset of transgenic plants; presumably those that disrupted EA1 expression, but this was not determined. Pollen tube guidance to *EA1* RNAi FGs was tested using in vitro pollination of ovules. In this assay, tubes entered the micropyle and burst within wild-type ovules 82% of the time. However,

this rate was reduced to 40–55% in ovules from two independent *EA1* RNAi lines. Pollen tubes were observed that grew past the micropyle in cases where ovule entry was unsuccessful. The EA1 expression pattern and the mild tube guidance phenotype in *EA1* RNAi plants are consistent with a function in the micropylar guidance phase. Therefore, EA1 meets two of the three criteria described above for a chemotropic guidance factor. Future studies will be aimed at determining whether EA1 functions directly as a chemotropic factor in vitro (Marton et al. 2005). It will also be interesting to clarify whether the mild impact on tube guidance is because EA1 expression was not completely eliminated in *EA1* RNAi plants, or whether this incomplete disruption of tube guidance indicates that there are redundant signals that direct pollen tubes to the micropyle in maize.

After fertilization, *EA1* mRNA was detected in the zygote for at least 45 hours but was not detected in 12-day-old embryos and EA1:GFP was eliminated rapidly from fertilized ovules (Marton et al. 2005). An interesting question will be to determine whether elimination of EA1 protein from the micropyle following fertilization is sufficient to prevent subsequent tubes from targeting the maize ovule or if a second repulsive signal is required.

5.5 Other Signals

While growing in the pistil, pollen tubes are exposed to many extracellular stimuli that could function as guidance cues. Several signals/molecules, including nutrients, have been reported to be involved in tube guidance including lipids, Ca^{2+} ions and cyclic nucleotides (Hülskamp et al. 1995; Malhó, this volume; Žárský et al., this volume). However, it is not clear if these molecules act as chemotropic signals.

Nitric oxide (NO) was recently suggested to reorient lily pollen tubes in vitro (Prado et al. 2004). In contrast to Chemocyanin and TTS, which behave as positive chemotropic factors, NO acted as a negative chemotropic factor, causing tubes to turn away from a point source. A gradient of NO was established by pipetting the NO donor, s-nitrosoacetylpenicillamine (SNAP), on to the surface of pollen growth medium. As tubes grew into the gradient, their growth rate slowed or stopped before the tip turned an average of 98° away from the point source. Interestingly, a mutant of Arabidopsis with a defect in NO production has reduced fertility, but tube guidance in this mutant has not been characterized (Guo et al. 2003). Rapid production of NO following entry of a tube into the micropyle could redirect subsequent tubes away from an ovule that is already being fertilized. It will thus be interesting to determine the sites of NO production within the pistil and to determine the path of pollen tubes growing within this and other NO synthesis mutants.

NO is known to regulate the production of the critical secondary messenger cGMP in animals. Sildenafil citrate causes accumulation of cGMP in

animal cells because it inhibits cGMP-degrading phosphodiesterases. Treatment of lily pollen tubes with sildenafil citrate sensitized the response to NO, suggesting that NO may also regulate cGMP production in pollen tubes. Interestingly, another cyclic nucleotide, cAMP has also been implicated in re-orientation of tip growth (Moutinho et al. 2001). Cyclic nucleotide synthesis is just beginning to be understood in plants (Malhó, this volume) and understanding the regulation of cyclic nucleotide signaling in pollen tubes will be an exciting area of future research.

6 The Search for Pollen Tube Receptors

How do pollen tubes recognize chemotropic guidance cues and transduce them into intracellular responses that result in changes in the direction of the tip? One hypothesis is that tubes express transmembrane receptors that specifically interact with guidance cues like those described above. Interaction between receptor and ligand is expected to initiate a signal transduction cascade within the tube that results in tip re-orientation and guidance (Malhó, this volume). It has been shown that localised $[Ca^{2+}]_c$ changes in the pollen tube apex control tube directioning (Malhó and Trewavas 1996). These changes may arise through asymmetric activity of putative Ca^{2+}-channels (Malhó et al. 1995; Sze et al., this volume). Preliminary evidence suggested the existence of low-voltage stretch-activated channels (Geitmann and Cresti 1998; Malhó et al. 1995) that could play a key role in the perception of guidance cues because of the multiple signalling pathways to which they respond (Ding and Pickard 1993).

Tremendous progress has been made in identifying intracellular factors that are crucial for tip growth (Malhó, this volume). For example, ROP proteins seem to act as a molecular switch that determines the site of cellular extension (Gu et al. 2005; Hwang and Yang, this volume). ROP modulates F-actin assembly and interferes with the dynamics of the tip-focused Ca^{2+} gradient, both of which are essential for tip growth and are thought to direct the flow of vesicles that ultimately results in polar tip extension (Malhó, this volume; Hepler et al., this volume; Yokota and Shimmen, this volume). Thus, changing the direction of tube growth may simply be a matter of changing the position/activity of certain proteins on the pollen tube plasma membrane. This has been demonstrated for several components like protein kinases, calmodulin, phosphoinositides and ROPs (Malhó et al. 2000; Malhó, this volume; Hwang and Yang, this volume; Žárský et al., this volume). Further characterization of these receptors for guidance cues and their modes of signal transduction within the pollen tube represent a very exciting challenge.

Genetic analysis of pollen tube-expressed genes required for guidance is an attractive experimental approach because it is not biased toward a pre-

conceived notion about how the tube perceives guidance cues and a thorough genetic analysis has the potential to identify genes that mediate multiple steps in signal transduction pathways. A loss of function mutation in a pollen tube-expressed guidance cue receptor putatively results in defective tube guidance that phenocopies loss of guidance cue production. For example, a tube that lacks the receptor for a micropylar guidance cue (perhaps EA1) would be expected to fail to enter the micropyle when grown in a wild type pistil the way wild type pollen tubes do when they grow toward an *ea1*- FG. Ideally, multiple mutants would be collected with defects in each of the specific phases of tube guidance described above and by identifying the disrupted genes in each group of mutants, hypotheses could be made about how the tube perceives cues that direct each phase of guidance. However, it is conceivable that such receptors act simultaneously as structural components of the tip growth machinery, and thus a mutation in their genes could result in the failure for pollen tubes to develop.

A large number of pollen tube mutants have now been characterized through a combination of reverse and forward genetic approaches (Twell et al., this volume; Guermonprez et al., this volume). Many of these affect tube germination and early stages of growth resulting in short tubes that fail to enter the ovary (Johnson et al. 2004; Lalanne et al. 2004; Guermonprez et al., this volume). These mutants have been useful for defining new mechanisms responsible for tip growth revealing a diverse set of biochemical processes that underlie tube extension. Several pollen mutants have also been characterized that do not affect germination, but disrupt tube growth; often these mutants have reduced growth rates that result in an inability to compete with wild-type tubes for access to ovules (Goubet et al. 2003; Johnson et al. 2004; Lalanne et al. 2004; Mouline et al. 2002; Schiott et al. 2004). Again, this group of mutants identified a diverse set of genes required for tube growth that include ion channels (Mouline et al. 2002; Schiott et al. 2004) and cell wall biosynthetic enzymes (Goubet et al. 2003). Despite growth defects, these mutant pollen tubes can target the ovules and are therefore able to respond to guidance cues along their growth path; thereby genetically separating the growth and guidance processes.

Pollen tube guidance mutants that have wild-type germination and growth rates, yet fail to target ovules, are rare. The *hapless* (*hap*) mutants are tagged with a pollen-specific marker gene that facilitates analysis of mutant pollen tube growth phenotypes within the ovary (Johnson et al. 2004). Out of 30 pollen mutants that were analyzed, 10 appeared to extend tubes that could grow the length of the pistil, yet these mutants showed reduced ability to target ovules. *hap1*, *hap18*, and *hap22* tubes failed to exit the transmitting tract; *hap11*, *hap26*, and *hap30* had relatively normal tube growth paths but were less likely than wild-type to enter the micropyle; and *hap2*, *hap4*, *hap24*, and *hap27* showed a chaotic growth path in the ovary and were often found growing on ovule surfaces where wild-type tubes do not grow (Johnson et al.

2004). So far, only a few of the genes disrupted in *hap* mutants have been identified and none of them appear to be similar to known receptors. Future work will be aimed at identifying more *hap* genes so that gene functions can be associated with specific defects in individual guidance steps.

Interestingly, pollen tube guidance mutants have yet not been found that completely block the ability of tubes to target ovules. This may suggest that there are redundant signaling mechanisms responsible for each phase of tube guidance and that no single gene mutation will completely disrupt guidance. Support for this idea comes from analysis of *ea1* mutants of maize and *myb98* and *pop2* mutants of *Arabidopsis* (Kasahara et al. 2005; Marton et al. 2005; Palanivelu et al. 2003; Wilhelmi and Preuss 1996). These mutants are proposed to disrupt production of chemotropic guidance cues, and all of them disrupt guidance: however, none of them completely block tube guidance.

A directed approach to identifying receptors for guidance cues is to study genes expressed by the tube that are homologous to known receptors. Microarray analysis of the 612-member receptor-like kinase (RLK) gene family in *Arabidopsis* showed that 10% are pollen expressed and that $\sim$ 90% of these are pollen specific (Honys and Twell 2003). This represents just one of several known receptor types expressed by pollen and illustrates the staggering potential diversity of signal transduction mechanisms in this cell. Functional characterization of members of the RLK gene family in tomato is well underway. LePRK1 and LePRK2 are pollen-specific RLKs that are localized to the surface of growing tubes (Muschietti et al. 1998). They both encode active kinases (Muschietti et al. 1998) and interact with each other at the pollen tube membrane; interestingly, this interaction and the phosphorylation of LePRK2 can be disrupted by incubation with a style extract in vitro (Muschietti et al. 1998; Wengier et al. 2003). This suggests that LePRKs may be involved in perception of extracellular growth regulators expressed by cells along the tube growth path.

Proteins that interact with the extracellular domain of LePRKs and are therefore putative ligands have been identified by yeast two hybrid screening of pollen [LAT52 (Tang et al. 2002)] and stigma cDNA libraries [LeSTIG1 and LeSHY (Tang et al. 2004)]. Loss of LAT52 function leads to pollen tube growth defects and LeSTIG1 has been shown to stimulate tube growth in vitro, suggesting that these two proteins may regulate growth via interactions with LePRKs. One interesting hypothesis is that the LePRK1/2 dimer switches ligands from LAT52 to LeSTIG1 upon contact with the stigma (Tang et al. 2004) and that this switch is critical for initiation of tube growth. One possibility to be tested is that LeSTIG1 is the active component of the stigma extract that causes LePRK1 and LePRK2 to dissociate and LePRK2 to become dephosphorylated.

To determine how signaling events at the pollen tube surface mediated by LePRKs are transduced into changes in tube growth, proteins that interact with intracellular domains of LePRKs were identified (Kaothien et al. 2005).

KPP is pollen-specific, phosphorylated in pollen, associated with pollen tube membranes, and its overexpression leads to defects in tip-localized actin dynamics that result in loss of apical polarity (Kaothien et al. 2005). All of these features implicate KPP in signal transduction events that regulate tube growth. Interestingly, KPP interacts with the cytoplasmic domains for LePRK1 and LePRK2 in vitro and this interaction does not require the kinase domains of either LePRK1or LePRK2. It is not yet known whether LePRK1 and/or LePRK2 mediate phosphorylation of KPP in pollen. In addition, it will be interesting to determine whether incubation with stigma extracts or LeSTIG1 alters the LePRK:KPP interaction or the phosphorylation status of KPP.

Analysis of LePRKs shows that taking a candidate-gene approach to the identification of receptors that sit atop pollen signal transduction pathways can be very productive. LePRKs have candidate ligands expressed by the pollen and stigma suggesting an autocrine/paracrine system for regulation of tip growth. Given the large number of candidate receptor genes expressed by pollen, more directed approaches will have to be taken to identify the specific receptor molecules that perceive guidance cues.

7
Are Guidance Factors Universal or Species Specific?

Pollen tube guidance cues and signal transduction pathways are being discovered in experimental model systems, and it will be extremely interesting to determine whether these systems are universal and function in all angiosperms or whether they are species specific. This is an important evolutionary question because diversification of tube guidance signals could represent a potent prezygotic barrier to interspecific fertility and may therefore be a means to achieve reproductive isolation following speciation. It is also an interesting question from an agricultural point of view because plant breeders would like to be able to make wider crosses between plants from different species but are sometimes hampered by incongruities in guidance systems.

There is ample experimental evidence indicating that important components of tube guidance mechanisms are species specific (Swanson et al. 2004). For example, pollen tubes of fellow Brassica family members like *Brassica sp.* and *Orychophragmus violaceus* fail to target *Arabidopsis* ovules in interspecific crosses (Kandasamy et al. 1994; Shimizu and Okada 2000). Pollen tubes germinate on the stigma and grow down the transmitting tract, but the majority of tubes failed to exit the transmitting tissue and those that did grew in the ovary in a fashion similar to tubes growing toward ovules lacking an FG (Ray et al. 1997; Shimizu and Okada 2000). Therefore, both the transmitting tract exit point signal (phase 3) and later signals mediating funicular

(phase 4) and micropylar guidance (phase 5) are likely to be species specific within the Brassicacae.

The guidance cues produced by *Torenia* and closely related genera are species specific in the in vitro guidance assay providing further evidence that the micropylar guidance signal is species specific (Higashiyama et al. 2003). While the chemical nature of the *Torenia* micropylar guidance cue is not yet known, the proposal that this signal is a polypeptide would accommodate species specificity due to rapid evolution and would also explain its limited effective range in vitro (Higashiyama et al. 2001). Species specificity could be achieved for a protein signal if the gene encoding the signal was subjected to positive selection following speciation and the receptors expressed by the pollen tube coevolved to more efficiently respond to the changed molecule. Interestingly, homologs for the proposed micropylar guidance cue from maize, the EA1 protein, were found only in rice, a fellow monocot, and alignment of EA1 proteins from maize and rice showed many amino acid changes especially at the N-terminus (Marton et al. 2005). It is possible that a small protein like EA1 serves as a micropylar guidance signal in all flowering plants, but rapid evolution during speciation has made EA1 analogs unrecognizable outside of grasses. Obvious EA1 homologs were not identified in the dicotyledonous *Arabidopsis* genome (Marton et al. 2005), however, the Arabidopsis genome encodes many small proteins that could represent the functional analog of EA1. Some crosses between maize and its ancestor teosinte are interfertile suggesting that micropylar guidance signals have not diverged since maize was domesticated (Baltazar et al. 2005); sequencing EA1 from teosinte and proximal relatives that are not interfertile will provide a test for the hypothesis that EA1 is rapidly diverging. In addition, identification of the synergid-derived signal that directs tube guidance in the dicot *Torenia* will reveal whether this signal could be an ancestor of EA1.

In contrast, a homolog of chemocyanin, the stigma guidance factor identified in the monocot lily, was identified in the dicot *Arabidopsis* (Dong et al. 2005). Furthermore, it was reported that plantacyanin from *Arabidopsis* can reorient lily tubes growing in vitro (Dong et al. 2005), suggesting that plantacyanins might be universal guidance cues. Overexpression of plantacyanin in *Arabidopsis* caused pollen tubes to coil and grow away from the style on papillae (Dong et al. 2005). These observations suggest that plantacyanins have a conserved function in orienting tube growth immediately after germination on the stigma. This conservation is particularly intriguing given the dramatically different architectures and chemical compositions of lily and Arabidopsis stigmas: In lily, tubes enter the style through pores in an exudate-rich wet stigma (Lord 2003); in *Arabidopsis*, tubes penetrate a papillar cell wall on a dry stigma (Elleman et al. 1992; Kandasamy et al. 1994).

Are small, structurally simple molecules, like GABA and NO universal guidance factors? Both of these molecules are produced by many organisms and are likely present in pistils of many if not all flowering plants. Despite

their ubiquity, a species-specific response could be mediated by quantitative differences in the amount of signal presented or in the sensitivity of the tube perception and response mechanisms. For example, co-evolution of the pollen tube and its host pistil following speciation could result in fine-tuning of GABA gradients and response pathways that would result in a species-specific response. Interestingly, when GABA levels are altered in *pop2* mutants, the tube growth path is very similar to that of *Brassica napus* or *Orychophragmus violaceus* tubes growing in an *Arabidopsis* pistil: most tubes fail to exit the transmitting tissue and those that do have defective funicular or micropylar targeting (Kandasamy et al. 1994; Palanivelu et al. 2003; Shimizu and Okada 2000).

The final phase of pollen tube guidance is reception (Phase 6), when the tube stops growing and bursts. In closely related, sympatric species without pre-pollination blocks to interfertility, this may be the last line of defense against interspecific fertilization. Characterization of the *feronia* and *sirene* mutants of *Arabidopsis* indicates that the FG produces a signal that controls this final phase and suggests that evolution of this signal could prevent tubes from other species from successfully delivering male gametes to the FG. Indeed, interspecific crosses in the genus *Rhododendron* are blocked after tubes enter the FG of the other species, but fail to stop growing and burst (Williams et al. 1986), much the same way wild-type tubes fail in *sirene* or *feronia* FGs (Huck et al. 2003; Rotman et al. 2003).

8 Perspectives

It is now clear that pollen tubes respond to chemotropic factors responsible for guidance in the pistil. Several phases of guidance have been delineated and multiple signals that act over different spatial ranges guide the pollen tube to the ovule. The sources of some of these signals within the pistil have been defined and chemotropic factors have been identified in a variety of angiosperm species using a combination of genetic, biochemical, and molecular approaches. The discovery of these molecules and their characterization marks tremendous progress in our understanding of guidance signals but many questions remain. Few of these molecules have satisfied all of the experimental criteria for a chemotropic guidance factor and none of them have been associated with pollen tube receptors or with the well-described signaling pathways within the tube that regulate tip growth. In addition, there are likely many more signals to discover and it will be very interesting to determine whether the signals defined thus far function in all plant species.

The field is now poised to define signal transduction pathways that link these extracellular guidance cues to intracellular networks that define, extend, and reorient the pollen tube tip. As these mechanisms are established

it will be exciting to determine the extent to which they are conserved among different plant species.

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References

Baker SC, Robinson-Beers K, Villanueva JM, Gaiser JC, Gasser CS (1997) Interactions among genes regulating ovule development in Arabidopsis thaliana. Genetics 145:1109–1124

Baltazar BM, Sanchez-Gonzalez JD, de la Cruz-Larios L, Schoper JB (2005) Pollination between maize and teosinte: an important determinant of gene flow in Mexico. Theor Appl Gen 110:519–526

Cheung AY, Wang H, Wu HM (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. Cell 82:383–393

Ding JP, Pickard BG (1993) Mechanosensory calcium-selective cation channels in epidermal cells. Plant J 3:83–110

Dong J, Kim ST, Lord EM (2005) Plantacyanin plays a role in reproduction in Arabidopsis. Plant Physiol 138:778–789

Elleman CJ, Franklin Tong V, Dickinson HG (1992) Pollination In Species With Dry Stigmas the Nature Of the Early Stigmatic Response and the Pathway Taken By Pollen Tubes. New Phytol 121:413–424

Geitmann A, Cresti M (1998) Ca^{2+} channels control the rapid expansions in pulsating growth of Petunia hybrida pollen tubes. J Plant Physiol 152:439–447

Goubet F, Misrahi A, Park SK, Zhang ZN, Twell D, Dupree P (2003) AtCSLA7, a cellulose synthase-like putative glycosyltransferase, is important for pollen tube growth and embryogenesis in Arabidopsis. Plant Physiol 131:547–557

Gu Y, Fu Y, Dowd P, Li S, Vernoud V, Gilroy S, Yang Z (2005) A Rho family GTPase controls actin dynamics and tip growth via two counteracting downstream pathways in pollen tubes. J Cell Biol 169:127–138

Guo FQ, Okamoto M, Crawford NM (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science 302:100–103

Heslop-Harrison J (1986) Pollen-Tube Chemotropism: Fact or Delusion. In: Cresti M, Dallai R (eds) Biol of reproduction and cell motility in plants and animals. Siena: University of Siena Press, Italy, p 169–174

Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. Int Rev Cytol 107:1–78

Higashiyama T, Kuroiwa H, Kawano S, Kuroiwa T (1998) Guidance in vitro of the pollen tube to the naked embryo sac of Torenia fournieri. Plant Cell 10:2019–2032

Higashiyama T, Kuroiwa H, Kuroiwa T (2003) Pollen-tube guidance: beacons from the female gametophyte. Curr Opin Plant Biol 6:36–41

Higashiyama T, Yabe S, Sasaki N, Nishimura Y, Miyagishima S, Kuroiwa H, Kuroiwa T (2001) Pollen tube attraction by the synergid cell. Science 293:1480–1483

Honys D, Twell D (2003) Comparative analysis of the Arabidopsis pollen transcriptome. Plant Physiol 132:640–652

Huck N, Moore JM, Federer M, Grossniklaus U (2003) The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception. Development 130:2149–2159

Hülskamp M, Schneitz K, Pruitt RE (1995) Genetic evidence for a long-range activity that directs pollen tube guidance in Arabidopsis. Plant Cell 7:57–64

Johnson MA, Preuss D (2002) Plotting a course: multiple signals guide pollen tubes to their targets. Dev Cell 2:273–281

Johnson MA, von Besser K, Zhou Q, Smith E, Aux G, Patton D, Levin JZ, Preuss D (2004) Arabidopsis hapless mutations define essential gametophytic functions. Genetics 168:971–982

Kandasamy MK, Nasrallah JB, Nasrallah ME (1994) Pollen-pistil interactions and developmental regulation of pollen tube growth in Arabidopsis. Development 120:3405–3418

Kaothien P, Ok SH, Shuai B, Wengier D, Cotter R, Kelley D, Kiriakopolos S, Muschietti J, McCormick S (2005) Kinase partner protein interacts with the LePRK1 and LePRK2 receptor kinases and plays a role in polarized pollen tube growth. Plant J 42:492–503

Kasahara RD, Portereiko MF, Sandaklie-Nikolova L, Rabiger DS, Drews GN (2005) MYB98 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis. Plant Cell 17(11):2981–2992

Kim S, Dong J, Lord EM (2004) Pollen tube guidance: the role of adhesion and chemotropic molecules. Curr Top Dev Biol 61:61–79

Kim S, Mollet JC, Dong J, Zhang K, Park SY, Lord EM (2003) Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. Proc Natl Acad Sci USA 100:16125–16130

Lalanne E, Michaelidis C, Moore JM, Gagliano W, Johnson A, Patel R, Howden R, Vielle-Calzada JP, Grossniklaus U, Twell D (2004) Analysis of transposon insertion mutants highlights the diversity of mechanisms underlying male progamic development in Arabidopsis. Genetics 167:1975–1986

Lord EM (2003) Adhesion and guidance in compatible pollination. J Exp Bot 54:47–54

Lush WM (1999) Whither chemotropism and pollen tube guidance? Trends Plant Sci 4:413–418

Malhó R, Camacho L, Moutinho A (2000) Signalling pathways in pollen tube growth and reorientation. Ann Bot 85(suppl A):59–68

Malhó R, Read ND, Trewavas AJ, Pais MS (1995) Calcium channel activity during pollen tube growth and reorientation. Plant Cell 7:1173–1184

Malhó R, Trewavas AJ (1996) Localised apical increases of cytosolic free calcium control pollen tube orientation. Plant Cell 8:1935–1949

Marton ML, Cordts S, Broadhvest J, Dresselhaus T (2005) Micropylar pollen tube guidance by egg apparatus 1 of maize. Science 307:573–576

Mascarenhas JP (1975) The biochemistry of angiosperm pollen development. Botanical Rev 41:259–314

Mollet JC, Kim S, Jauh GY, Lord EM (2002) Arabinogalactan proteins, pollen tube growth, and the reversible effects of Yariv phenylglycoside. Protoplasma 219:89–98

Mollet JC, Park SY, Nothnagel EA, Lord EM (2000) A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix. Plant Cell 12:1737–1750

Mouline K, Very AA, Gaymard F, Boucherez J, Pilot G, Devic M, Bouchez D, Thibaud JB, Sentenac H (2002) Pollen tube development and competitive ability are impaired by disruption of a Shaker $K(^{+})$ channel in Arabidopsis. Genes Dev 16:339–350

Moutinho A, Hussey PJ, Trewavas AJ, Malhó R (2001) cAMP acts as a second messenger in pollen tube growth and reorientation. Proc Natl Acad Sci USA 98:10481–10486

Muschietti J, Eyal Y, McCormick S (1998) Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2. Plant Cell 10:319–330

Pagnussat GC, Yu HJ, Ngo QA, Rajani S, Mayalagu S, Johnson CS, Capron A, Xie LF, Ye D, Sundaresan V (2005) Genetic and molecular identification of genes required for female gametophyte development and function in Arabidopsis. Development 132:603–614

Palanivelu R, Brass L, Edlund AF, Preuss D (2003) Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. Cell 114:47–59

Pinal CS, Tobin AJ (1998) Uniqueness and redundancy in GABA production. Perspect Dev Neurobiol 5:109–118

Prado AM, Porterfield DM, Feijó JA (2004) Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development 131:2707–2714

Ray SM, Park SS, Ray A (1997) Pollen tube guidance by the female gametophyte. Development 124:2489–2498

Rotman N, Rozier F, Boavida L, Dumas C, Berger F, Faure JE (2003) Female control of male gamete delivery during fertilization in Arabidopsis thaliana. Curr Biol 13:432–436

Sanders L, Lord E (1989) Directed movement of latex particles in the gynoecia of three species of flowering plant. Science 243:1606–1608

Schiott M, Romanowsky SM, Baekgaard L, Jakobsen MK, Palmgren MG, Harper JF (2004) A plant plasma membrane Ca^{2+} pump is required for normal pollen tube growth and fertilization. Proc Natl Acad Sci USA 101:9502–9507

Shimizu KK, Okada K (2000) Attractive and repulsive interactions between female and male gametophytes in Arabidopsis pollen tube guidance. Development 127:4511–4518

Swanson R, Edlund AF, Preuss D (1994) Species specificity in pollen-pistil interactions. Annu Rev Gen 38:793–818

Tang W, Ezcurra I, Muschietti J, McCormick S (2002) A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2. Plant Cell 14:2277–2287

Tang W, Kelley D, Ezcurra I, Cotter R, McCormick S (2004) LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth in vitro. Plant J 39:343–353

Wengier D, Valsecchi I, Cabanas ML, Tang WH, McCormick S, Muschietti J (2003) The receptor kinases LePRK1 and LePRK2 associate in pollen and when expressed in yeast, but dissociate in the presence of style extract. Proc Natl Acad Sci USA 100:6860–6865

Wilhelmi LK, Preuss D (1996) Self-sterility in Arabidopsis due to defective pollen tube guidance. Science 274:1535–1537

Williams EG, Kaul V, Rouse JL, Palser BF (1986) Overgrowth of pollen tubes in embryo sacs of Rhododendron following interspecific pollinations. Aust J Bot 34:413–423

Wu HM, Wang H, Cheung AY (1995) A pollen tube growth stimulatory glycoprotein is deglycosylated by pollen tubes and displays a glycosylation gradient in the flower. Cell 82:395–403

Yadegari R, Drews GN (2004) Female gametophyte development. Plant Cell 16:S133–141