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Fertilization Requires Communication: Signal Generation and Perception During Pollen Tube Guidance

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ABSTRACT

The generation of novel hybrid lines is critically limited by the compatibility between pollen and pistil and their ability to achieve successful seed formation. Fertilization in flowering plants requires the successful transfer of the male gametes from the pollen grain to the egg contained within an ovule. This occurs via the formation of a tubular protrusion from the pollen grain – the pollen tube. This specialized structure grows extremely fast and has the complex task to invade the stigmatic and stylar tissues of the receptive flower, to find the ovary and an ovule, and to subsequently release the male gametes to the egg and central cell to achieve double fertilization. Failure of the pollen tube to penetrate the pistil or find its target results in the absence of fertilization and sterility. Understanding the signals that regulate the compatible interaction between a pollen tube and all the female cells in its path is therefore critical to generate tools for breeders in their quest to break species barriers and produce novel hybrids. In this review, we will discuss the signals and cues that guide pollen tubes to their targets, the mechanism of pollen tube growth and how the pollen tube readjusts its growth direction in response to these signals. In addition, we will present *in vitro* experimental strategies to study the pollen tube's ability to find its target and/or the ovule's capacity to attract or repulse a growing pollen tube.

Keywords: attraction, capacitance, compatibility, guidance, ovule, pollen tube, repulsion, sexual plant reproduction, signal transduction

CONTENTS

PATHFINDING IN PISTILS

During compatible pollination, the first challenge for a sperm-bearing pollen is to reach the female reproductive structure, the pistil, and attach to its landing platform, the stigma. Subsequently, to achieve fertilization, the pollen has to deliver the sperm to the egg, which is typically located further away from the stigma and buried deeply within pistil tissues. To achieve this, the pollen grain forms a protuberance that invades the stigma and through which the sperm cells are transported (Weterings and Russell 2004). On their way through the pistil, these pollen tubes migrate past several different cell types, growing between the walls of the stigma cells, travelling through the extracellular matrix of the transmitting tissue, and finally arriving at the ovary, where they migrate up the funiculus, and enter the ovule through an opening, the micropyle, to fertilize an egg and a central cell (**Fig. 1**; Lord and Russell 2002). Typically, only one pollen tube enters the ovule through the micropyle, terminates its journey within a synergid cell, and bursts to release two sperm cells, one of which fuses with the egg cell to form an embryo and the other merges with the central cell to generate an endosperm (Huck *et al.* 2003). Besides the complexity arising out of cell-cell communication, the pollen tube also has to traverse a long path (several centimeters in large flowers; Herrero and Hormaza 1996) and be able to sustain growth over a considerable amount of time (Sogo and Tobe 2006) before it reaches the ovule micropyle. In this chapter, we will review the knowledge gained on understanding the guidance cues produced by the female pistil tissues and the responses they elicit in the pollen tube.

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Invited Review

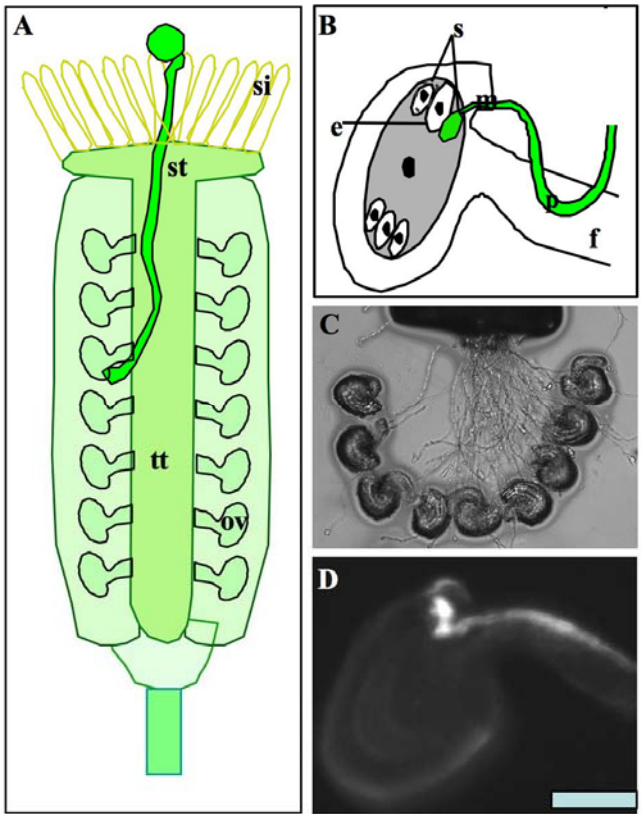

Fig. 1 Pollen tube guidance to ovules. (**A**) Diagram of a pollinated pistil within a flower. After reaching the stigma (si), pollen (green) extends a tube through the style (st) to reach the transmitting tract (tt) before entering the ovary chambers to target an ovule (ov). (**B**) Upon reaching the ovule, the pollen tube (p, green) either grows up the funiculus (f) or makes a sharp turn towards the micropyle (m) and enters the ovule. Within the ovule, the pollen tube navigates towards the female gametophyte (gray), lyses within one of the two synergid (s) cells that flank an egg cell (e). Upon lysis, one sperm fertilizes the egg cell to form the zygote and the other fuses with the central cell (c) to form the endosperm. (**C**) Bright field image depicting the *Arabidopsis thaliana in vitro* pollen tube guidance assay. Pollen tubes emerge from the cut portion of the pistil, travel across the agarose medium before reaching the excised ovules. (**D**) Fluorescent image of an ovule successfully targeted by a GFP-tagged pollen tube in the *in vitro* assay. The spot within the ovules marks successful pollen tube targeting. Scale bar, 100 μm.

THE FEMALE PERSPECTIVE

Signals that regulate pollen tube growth to the ovary

A combination of genetic, biochemical and *in vitro* assays have defined signals that contribute to the early stages of pollen tube guidance. The early phase of pollen tube growth – germination and penetration of the stigma – is influenced by the stigma exudate in plants with wet stigmas (e.g. tobacco) and by the pollen coat in species with dry stigmas (e.g. *Arabidopsis*). Lipids in the exudate and the pollen coat are required for pollen germination (Preuss *et al*. 1993; Wolters-Arts *et al*. 1998), perhaps functioning by controlling the flow of water to pollen (Lush *et al*. 1998). Chemocyanin, a small basic protein from lily stigmas, attracts lily pollen tubes *in vitro* (Kim *et al*. 2003). Wild type pollen tubes growing on stigmas overexpressing the *A. thaliana* chemocyanin homolog made numerous turns around the stigma before growing toward the style; this pollen tube behavior is in stark contrast to those growing on wild type stigmas when pollen tubes travel down in a targeted way from the stigma to the style (Dong *et al*. 2005).

Successful germination and growth of pollen tube through stigmas is followed by entry into the style tissue and eventually penetration of the transmitting tissue, where a nutrient-rich extracellular matrix that is secreted by the female pistil cells supports pollen tube growth. In *A. thaliana No Transmitting Tract* (NTT) gene, a C2H2/C2HC zinc finger transcription factor that is expressed specifically in transmitting tissue, increases fertilization efficiency in the bottom half of the ovary; disruption of this gene resulted in seed set only in the upper half of the ovary (Crawford *et al*. 2007). In combination with pectin a 9 kd lipid transfer protein has been shown to mediate adhesion of pollen tubes to the stylar matrix in lily (Mollet *et al*. 2000; Park *et al*. 2000). Two *Arabidopsis* Auxin Response Factors, *ARF6* and *ARF8*, regulate gynoecium and stamen development in immature flowers. Wild-type pollen grew poorly in *arf6 arf8* gynoecia, correlating with ARF6 and ARF8 expression in style and transmitting tract (Wu *et al*. 2006). Few candidate molecules that play a role in pollen-pistil signaling have been characterized in the transmitting tissue; nonetheless, the abundance of arabinogalactans in the pistil has prompted several investigations of these components (Jauh and Lord 1996). Arabinogalactan proteins (AGPs) play a critical role in cell-cell signaling and cell recognition during pollination. Support for this hypothesis comes from observations that phenylglycosides that specifically bind and precipitate AGPs also inhibit pollen tube growth when injected into transmitting tissues (Jauh and Lord 1996). Furthermore, the AGPs *TTS1* and *TTS2* from *Nicotiana tabacum* and *NaTTS* from *N. alata* are localized to the transmitting tract extracellular matrix and stimulate pollen tube growth *in vitro* (Cheung 1995; Wu *et al*. 2000). Although potential homologs of these proteins exist in *A. thaliana*, their role in pollen tube growth is yet to be determined (Wu *et al*. 2000). Transgenic tobacco plants expressing antisense *TTS1* and *TTS2* mRNA are sterile (Cheung 1995; Wu *et al*. 1995). These TTS proteins display a gradient of increasing glycosylation that correlates with the direction of pollen tube growth; both *in vitro* and *in vivo* studies indicate that the TTS proteins (Wu *et al*. 1995) and another AGP, *NaPRP5* (Lind *et al*. 1994), are incorporated into pollen tubes where they are deglycosylated.

Multiple signals guide pollen tubes to the ovule micropyle

After emerging from the transmitting tract, pollen tubes approach the ovule micropyle with remarkable precision (**Fig. 1B**). Similar to the early stages, it is apparent that multiple signals are important for proper pollen tube guidance in the ovary as well (Herrero 2001). Mutants with severe lesions in ovule development have an extreme pollen tube guidance phenotype and include *bel1* (Hülskamp *et al*. 1995), *sin1* (Schneitz *et al*. 1997), *ant* (Elliott *et al*. 1996), and *tso1* (Hauser *et al*. 1998) in *A. thaliana* and ACC oxidase mutants in tobacco (de Martinis and Mariani 1999). Because these mutants disrupt both haploid and diploid tissues, further analysis was required to dissect the source of pollen tube guidance signals. Diploid cells of an ovule also have a role in pollen tube guidance; female sporophytic *A. thaliana* mutants *ino* (Baker *et al*. 1997) and *pop2* (Palanivelu *et al*. 2003) have apparently normal female gametophytes yet pollen tubes do not grow normally to the mutant ovules (**Fig. 2**).

Several lines of evidence have established that the haploid female gametophyte is also essential for normal pollen tube guidance (**Fig. 2**). By reciprocal chromosomal translocation approach, pistils in which half the ovules contained apparently normal diploid tissues yet lacked a haploid female embryo sac were generated (Ray *et al*. 1997). When these pistils were pollinated, pollen tubes correctly targeted only the ovules with normal but not those with aberrant embryo sacs. In *A. thaliana maa* mutants (Shimizu and Okada 2000), the two polar nuclei of the embryo sac fail to fuse and as a result, a central cell is not formed. In these mutants, the pollen tubes adhere and grow up on the funiculus normally but fail to enter the micropyle. Based on these studies,

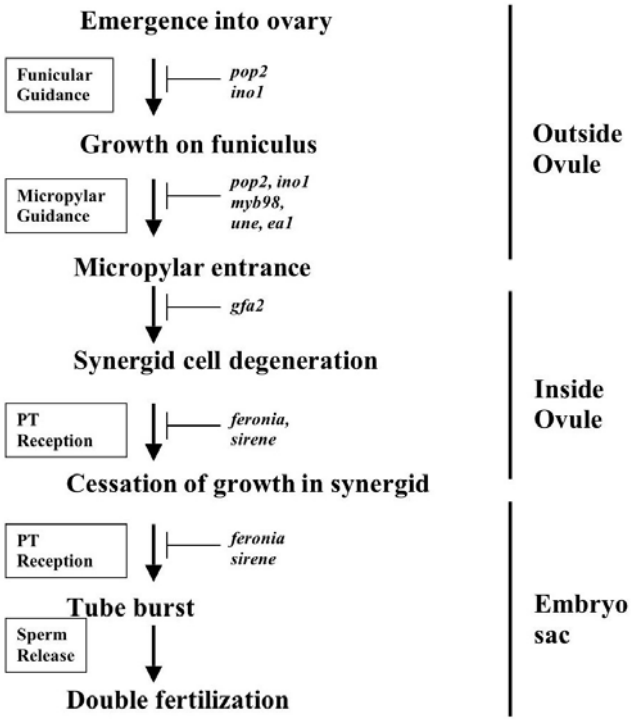

Fig. 2 Hallmark events during and mutants affecting pollen tube guidance within an ovule.

it was proposed that final stages of pollen tube growth can be divided into two distinct phases: (*i*) funicular guidance, in which pollen tubes adhere to and grow up the funiculus; this phase is likely mediated by diploid tissues of the ovule, and (*ii*) micropylar guidance, mediated by signals from the female gametophyte that guide pollen tube entry into the micropyle to deliver sperm to the female gametophyte (Shimizu and Okada 2000). Micropylar guidance signals originate at least in part from the two synergid cells contained within the female gametophyte. This conclusion is based on the studies which showed that pollen tubes do not enter ovules in which synergid cells were either ablated by laser (Higashiyama *et al*. 2001, 2006) or defective due to a T-DNA insertion in the *MYB98* gene (Kasahara *et al*. 2005). The recent identification of a battery of synergid-expressed genes that are directly regulated by *MYB98* (Punwani *et al.* 2007), offers an important opportunity to identify the chemoattractant that directs micropylar guidance. The maize EA1 protein, which is exclusively expressed in the egg and synergids of unfertilized female gametophytes, also has a role in regulating micropylar guidance. Plants expressing EA1 RNAi or antisense constructs produced significantly fewer seeds than wild type, and wild type pollen tubes failed to enter mutant ovules (Marton *et al*. 2005). *A. thaliana une* mutants despite containing apparently normal ovules exhibited defective micropylar guidance (Pagnussat *et al*. 2005); characterization of these mutants and those in recently identified female gametophyte-specific genes (Steffan *et al.* 2007) should unravel additional late guidance events (Pagnussat *et al*. 2005).

Pollen tube guidance within an ovule

After entering the ovule micropyle, pollen tubes navigate past diploid cells (inner and outer integument layers and the nucellus), grow through the filiform apparatus, reach one of the synergid cells, stop growing and burst to release the two sperm cells, a process termed pollen tube reception (**Fig. 2**; Huck *et al*. 2003). In many species including *A. thaliana*, the synergid cell that will ultimately receive the pollen tube degenerates either at the time of pollen tube entry into the ovule or shortly thereafter (Faure *et al*. 2002). Consistent with this prediction, using a variety of microscopic approaches, it was demonstrated that synergid cell death initiates after the pollen tube arrives at the female gametophyte but before pollen tube discharge (Sandaklie-Nikolova *et al.* 2007). Evidence suggests that synergid cell degeneration is not essential for pollen tube reception, however. This conclusion is based on studies with *gfa2* mutant ovules in which pollen tube reception is normal even though synergid cells do not degenerate (Christensen *et al*. 2002). Analysis of *A. thaliana sirene* and *feronia* female gametophytic mutants clarified which portion of an ovule is important for pollen tube reception. Within these mutant ovules pollen tube reception did not occur, instead pollen tubes continued to grow within the embryo sac, similar to the observations made with incompatible crosses involving several species within the *Rhododendron* genus (Williams *et al.* 1982). These results suggested that a functional female gametophyte is essential for pollen tube reception (Huck *et al*. 2003; Rotman *et al*. 2003). Recently, it was shown that *feronia* and *sirene* are allelic mutations in a receptor-like kinase gene that is expressed in synergid cells (Escobar-Restrepo *et al.* 2007). A fully differentiated filiform apparatus is not essential for pollen tube reception as filiform apparatus development is defective in *myb98* ovules, yet pollen tube reception is normal within these mutant ovules (Kasahara *et al*. 2005).

Pollen tube repulsion

Characterizations of pollen tube growth near an ovule have typically shown only one pollen tube emerging from the septum, climbing up the funiculus and entering the micropyle. It is also known that additional pollen tubes, despite coming close to the micropyle, do not enter fertilized ovules in the *Torenia fournieri* (wishbone flower) *in vitro* assay (Higashiyama *et al*. 1998). These results, however, did not clarify if additional tubes failed to enter an ovule due to termination of ovule attractant release or alternatively, that they were actively repelled by a new signal. The female gametophyte has a role in repelling pollen tubes as evidenced by multiple pollen tubes either associating with *maa* (Shimizu and Okada 2000) and *myb98* ovules (Kasahara *et al*. 2005) or gaining entry into *sirene* (Rotman *et al*. 2003) and *feronia* ovules (Huck *et al*. 2003). Even though multiple pollen tubes entered *feronia* ovules, such supernumerary pollen tube behavior was observed only in ~10% of ovules, suggesting that additional mechanisms must exist to repel pollen tubes from ovules already penetrated by a pollen tube. As described below, an *A. thaliana in vitro* pollen tube guidance assay recapitulates this *in vivo* pollen tube repulsion behavior; multiple pollen tubes were denied access into a targeted ovule micropyle by a rapidly activated short-range pollen tube repulsion mechanism (Palanivelu and Preuss 2006). Based on these results we propose that, as a first line of defence, pistils prevent multiple pollen tubes from even associating with a funiculus of an ovule (long-range pollen tube repulsion mechanism; **Fig. 2**). In the event that this block breaks down or is removed, as witnessed in female gametophyte mutants and in the *A. thaliana in vitro* guidance assay, respectively, a second short-range repulsion mechanism, originating from the ovule, prevents additional tubes from entering the fertilized ovule (**Figs. 2** and **3**).

Despite the existence of such prevention mechanisms, multiple tubes still enter an ovule. In maize, heterofertilization results when the egg and central cell are fertilized by different pollen tubes at a frequency of ~2% (Kato 2001) and in *A. thaliana*, ~1% (Huck *et al*. 2003). Based on these natural occurrences several models have been put forward to explain why pollen tube repulsion is initiated. The first model suggests that pollen tube repulsion is essential to prevent intra genomic conflicts that would rise from egg cell and central cell being fertilized by genetically distinct sperm (Grossniklaus and Schneitz 1998). The second theory is that pollen tube repulsion prevents multiple pollen tubes from growing to the nearest ovule and instead facilitates

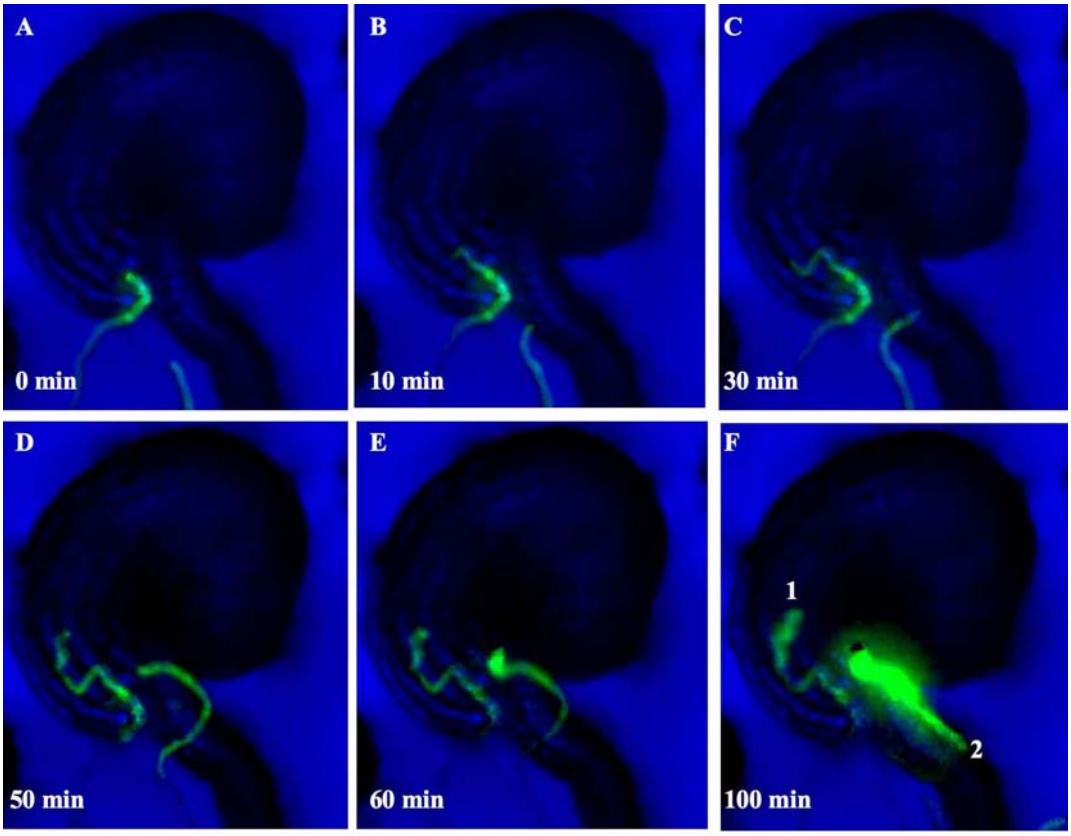

Fig. 3 Ovule-dependent short-range repulsion in the *Arabidopsis thaliana* guidance assay. (**A–F**) Merged fluorescent and bright field images of behavior of two pollen tubes near an ovule in the *in vitro* guidance assay. Two pollen approach an ovule; pollen tube #1 enters the ovule; the late arriving pollen tube #2 does not gain access into the ovule; instead it grows on the ovule and eventually climbs back down the funiculus. Elapsed time in minutes; Scale bar, 100 μm.

fertilization of sibling ovules and increases inclusive fitness, similar to the selfless behavior of haploid female worker bees that help their close relatives (Shimizu and Okada 2000).

In vitro assays facilitate characterizations of pollen tube behavior to extracellular cues

To identify agents that are able to influence the direction of pollen tube growth, pollen tubes are generally grown *in vitro* within or on a medium that contains a gradient of the substance in question. A redirection of growth towards or away from a point-source of a substance provides important information on the nature and optimal concentration of pollen tube attractants and agents inducing repulsion (Reger *et al*. 1992; Prado *et al*. 2004). In a more sophisticated approach, excised ovules are positioned in the *in vitro* setup and thus the cells within the ovule that produce the attracting agents can be identified. Two species in which this technically not trivial approach has been standardized successfully are *T. fournieri* and *A. thaliana*.

T. fournieri system: In *T. fournieri*, pollen tube guidance across a simple medium and into the ovule was achieved only after pollen tubes were grown through a stigma and style (Higashiyama *et al*. 1998). In this species, the female gametophyte protrudes from the ovule, and pollen tubes enter the micropyle without interacting with the funiculus (Higashiyama *et al*. 1998). Thus, the *T. fournieri in vitro* guidance system serves as a model for the micropylar, but not the funicular guidance phase of pollen tube growth to ovules. Using this system, it was demonstrated that when synergid cells were ablated, pollen tubes did not penetrate the micropyle, demonstrating that the synergid is the source of the attractant that facilitates pollen tube entry into the micropyle (Higashiyama *et al*. 2001).

Arabidopsis thaliana system: The pollen tube growth and guidance system in *A. thaliana* is amenable to genetic, biochemical and cell biological techniques and therefore is an ideal model system to decipher numerous aspects of this process during plant reproduction. Recently, an *A. thaliana in vitro* guidance assay that recapitulates both funicular and micropylar guidance phases of pollen tube growth to ovules was developed (Palanivelu and Preuss 2006). Previous studies in the *Torenia* system indicated that pollen tubes germinated on a simple growth medium cannot be guided to the micropyle (Higashiyama *et al*. 1998, 2001). As a result, a semi-*in vivo* situation was created by removing the lower portion of the pistil leaving only stigma and the upper part of the style (Cheung 1995; Higa-shiyama *et al*. 2001; Kim *et al*. 2003; Palanivelu *et al*. 2003). Pollen was deposited on the stigmatic surface and pollen tubes emerged from the cut end of the style, travelled across an agarose medium to excised ovules and successfully entered the micropyle (**Figs. 1C, 1D**). To facilitate pollen tube observation, especially after they enter the micropyle and are obscured by the opaque ovule integument cells, pollen tagged with GFP expressed from a pollen-specific LAT52 (LATE ANTHER TOMATO52) promoter (Twell *et al*. 1989) were used. Upon reaching the female gametophyte, these tubes ceased growth, burst and released a large spot of GFP (**Fig. 1D**), conveniently marking successfully targeted ovules. Pollen tubes that grew within ~100 μm of an unfertilized ovule often made a sharp turn toward the ovule; of the tubes that grew within this range, ~50% successfully entered the micropyle (Palanivelu and Preuss 2006).

Torenia and A. thaliana in vitro guidance assays demonstrate that the stigma and style capacitate pollen tubes to target ovules

With a ~50% efficiency the ovule targeting typically obtained in the semi-*in vivo* set up is significantly higher than that by tubes germinated on agarose (~3%). Thus, pollen tubes acquire the ability from pistil tissue to perceive ovule guidance signals, perhaps by absorbing essential nutrients or undergoing critical developmental transitions by physically interacting with the pistil tissues; a similar phenomenon was reported in *T. fournieri* (Higashiyama *et al*. 1998). This pistil-induced pollen tube competence is functionally analogous to the transformation that mammalian spermatozoa undergo after residence for a finite amount of time in the reproductive tract (Chang 1951; Austin 1952). Because of the functional similarity between the two processes, we propose that the pollen tube transformation in the pistil be

referred to as "pollen tube capacitance". In the *A. thaliana* system, sometimes pollen grains that germinated on the stigma formed tubes that grew onto the medium, rather than penetrating the pistil and growing through the style. Nonetheless, these tubes successfully targeted excised ovules (19%), suggesting that interaction with the stigma alone is sufficient to capacitate pollen tubes (Palanivelu and Preuss 2006).

Ovule-based pollen tube repulsion signaling

When multiple pollen tubes migrated near an ovule in the *Torenia* and *A. thaliana in vitro* guidance system, only one pollen tube gained access to each micropyle (Higashiyama *et al*. 1998; Palanivelu and Preuss 2006). These results are similar to observations made *in vivo*; typically only one tube migrates up the funiculus and gains entry into the ovule (Shimizu and Okada 2000). To consistently monitor pollen tube repulsion, the *A. thaliana in vitro* assay was modified by having only a minimal number of tubes emerge from the style facilitating easy observation of the attraction of successful tubes and repulsion of late arriving tubes.

Repulsion near an ovule could be either because ovule attraction terminates after fertilization, or alternatively, that a new signal repels additional pollen tubes. To distinguish between these possibilities, time-lapse imaging analysis of targeted ovules that were approached by more than one pollen tube was used (**Fig. 3**). Pollen tube repulsion occurred on an average 27 μm from the micropyle (Palanivelu and Preuss 2006). In addition, repelled tubes stalled near the micropyle (Palanivelu and Preuss 2006) or turned sharply away from the targeted ovule (**Fig. 3**, tube # 2). These responses were observed as early as 10 minutes after a successful targeting event, i.e. the unsuccessful tubes actively approached the ovule, yet suddenly changed course near the micropyle of an ovule that was just penetrated by another tube (compare **Fig. 3A** and **3B**). Together these behaviors strongly suggest that an active, short-range repulsion mechanism exists to prevent multiple pollen tubes from gaining access to an ovule. Time-lapse observations using the *in vitro* assay also indicated that, (*i*) repulsion responses occurred well before the pollen tube reached the female gametophyte and released its cytoplasm within the ovule, suggesting tube burst is not required for the initiation of repulsion signaling; (*ii*) repulsion initiated soon after a successful pollen tube interacted with the ovule diploid integument cells and considerably before they reached the haploid female gametophyte, suggesting that diploid cells play a role in generation of repulsion signals (Palanivelu and Preuss 2006) and that repulsion initiates prior to tube reception in the female gametophyte.

Multiple pollen tubes gain access to *sirene* and *feronia* mutant ovules but fail to undergo pollen tube reception and burst (Huck *et al*. 2003; Rotman *et al*. 2003). Based on this it was proposed that pollen tube repulsion does not initiate until these events occur. On the contrary, in the *A. thaliana in vitro* guidance assay it was observed that pollen tube repulsion responses initiate well before tube reception/burst occurs, establishing that tube burst is not a prerequisite for repulsion. The dramatic re-orientation exhibited by repelled tubes is strikingly similar to the sharp turning behavior of lily pollen tubes when exposed to nitric oxide (NO; Prado *et al*. 2004). It remains to be confirmed whether NO is the pollen tube signal from ovules. Since short-range repulsion in the *A. thaliana in vitro* system is rapidly activated upon pollen tube interaction with ovule cells, it is likely that NO diffuses only from a targeted ovule to repel pollen tubes. Given that *A. thaliana* mutants in nitric oxide synthase have been recently identified (Guo *et al*. 2003), and that the *in vitro* guidance assay facilitates easy collection of recently targeted ovules that has been nearly impractical until now, it should be now possible to identify and characterize repellent signals from ovules.

Pollen tube guidance is regulated by species-specific signals

Inter specific hybridization is central to floral variety improvement as it provides a way to generate new allele combinations and introduce desirable traits into the breeding stocks. Novel hybrid flowers are also commercially desirable because of their enhanced yield, coloring pattern and size. However, in many instances inter-specific hybridizations are not feasible due to incompatibility barriers, some of which occur during pollen tube growth and guidance on a pistil. For example, interspecies incompatibility barrier in lily caused due to poor pollen tube growth in style was overcome by grafting a compatible style onto an incompatible ovary (Roggen *et al*. 1988). Cytological observations of incompatible crosses among selected genus of *Rhododendron* and *Arabidopsis* have revealed that pollen tubes exhibit several type of abnormalities, including the coiling behavior in the embryo sac (similar to those caused by *feronia* and *sirene* mutations), in seven different parts of the pistil (Williams *et al*. 1982; Escobar-Restrepo *et al.* 2007). Alternatively, *in vitro* guidance assays such as those described for *Torenia* and *A. thaliana* can pinpoint which tissues are sufficient for successful pollination and bypass the tissue that acts as a incompatibility barrier as observed for interactions between L. c. pollen and *Torenia fournieri* pistil (compare Figs. 4 and 8 in Higashiyama *et al*. 2006). Based on this example, in lily interspecific crosses, style tissue can be avoided. Besides, the *in vitro* assay can help characterize and enhance our understanding of the interspecies pollination interactions.

To explore the variability among the short-range ovule attractants, pollen and pistil tissues from *A. thaliana* were used in the *in vitro* guidance assay while sampling ovules from close relatives of *Arabidopsis* (Hall *et al*. 2002). Significantly reduced targeting of *A. thaliana* pollen to *A. arenosa* and *Olimarabidopsis pumilla* ovules was observed and no targeting to *Capsella rubella* and *Sisymbrium irio* ovules was found (Palanivelu and Preuss 2006). These results show that the guidance signals which direct tube entry into ovules are rapidly evolving even among closely related species of *Arabidopsis*. Similarly, when five closely related species in two genera, namely *Torenia* and *Lindernia*, were used in the *Torenia in vitro* guidance system, it was found that pollen tubes preferentially migrated to synergid cells of their own species than to other species. In addition, *in vivo* crossing experiments also showed that embryo sacs of one species did not attract pollen tubes of different species even if these reached their ovule micropyles (Higashiyama *et al*. 2006). These results strongly suggest that the micropylar guidance step may serve as a reproductive barrier during pollen migration to an ovule. Contrary to the rapid divergence of the micropylar entry signals, capacitance signals seem to evolve less rapidly among different species. In the *A. thaliana in vitro* guidance assay, when pollen and ovules from stage 14 pistils were sampled with stigma and style from different development stages or close relatives of *A. thaliana*, it was observed that pollen tube targeting efficiency increased two fold between stages 10 and 14, and then declined to a small extent by stage 18 (Palanivelu and Preuss 2006). Efficiency with which *A. thaliana* pollen tubes targeted its own ovules was invariable whether it grew through its own or through *A. arenosa*, *Olimarabidopsis pumilla* and *Sisymbrium irio* pistils (Palanivelu and Preuss 2006). Similar observations were made for many members of the Scrophulariaceae (Higashiyama *et al*. 2006).

THE MALE PERSPECTIVE

For a pollen tube to react to a cue presented by the pistil or the female gametophyte three steps must occur: signal perception, signal transduction and cellular response. All of these steps require the presence and functioning of the respective molecular machinery, the importance of which is illustrated by the abundance of mRNAs coding for proteins

involved in signal transduction in pollen (Becker *et al.* 2003; Honys and Twell 2003). In this context it is interesting to note, that proteins expressed in the sperm cells also seem to play a role in pollen tube guidance (von Besser *et al*. 2006), but it is unknown as of now whether they are implied in cellular signal transduction or in the response.

The nature of the cues eliciting these intracellular signaling steps varies between species and depends on the location within the pistil. The *in vitro* testing of pollen tube tropism to pistil parts and extracts and to various other agents has a long history (for reviews see Mascarenhas 1978; van Went and Willemse 1984; Heslop-Harrison 1987; Vasil 1987; Mascarenhas 1993; Lord and Russell 2002). Pollen tubes were observed to be able to react to a variety of signals; in particular their ability to exhibit chemotropism, thigmotropism, and electrotropism *in vitro* has been studied. It has to be considered, however, that while the demonstration of a tropic behavior towards an agent in an *in vitro* setup is an indicator, it is by no means a proof, that this mechanism is effective *in planta*.

Signal perception

Signal perception proper implies the contact of a signal molecule with a cellular structure or another immediate effect of the signaling cue on the cell. The structures involved in signal perception depend on the nature of the signal which can be physical, chemical or electrical. Physical signals in the style can consist of mechanical barriers in the form of a stigmatic cuticle or the cell walls of the transmitting tissue. The availability of water (Lush *et al*. 1998; Wolters-Arts *et al*. 1998) can probably also be classified as a physico-chemical cue. Chemical cues comprise the availability of nutrients and ions but also specific signaling molecules. Among these several have been shown to induce chemotropism in pollen tubes grown *in vitro*: The transmitting tissue-specific protein TTS in tobacco (Cheung *et al*. 1995; Wu *et al*. 1995, 2000), chemocyanin (Kim *et al*. 2003) and stigma/stylar Cys-rich adhesin and pectin in lily (Mollet *et al*. 2000; Park and Lord 2003), and a very acidic ovarian protein in *Pennisteum glaucum* (Reger *et al*. 1992). Other molecules that have been proposed to be involved in pollen tube guidance include γ-aminobutyric acid (Palanivelu *et al*. 2003) and ZmEA1, a protein expressed in the maize egg cell and synergid cells (Marton *et al*. 2005). These molecules probably act through contact with a pollen tube receptor, which in turn triggers an intracellular signaling cascade. Not all protein molecules emitted from the stigma and acting on the pollen tube necessarily trigger signaling. In the S-RNase based gametophytic self-incompatibility system the effect on pollen tube growth is based on the enzymatic and therefore cytotoxic activity of the protein (Cruz-Garcia *et al*. 2003).

The nature of the pollen tube receptors to which signaling molecules bind is mostly unknown. However, several members of the largest group of plant receptor kinases, the LRR (leucine-rich repeat) kinases have been found to be pollen-specific (Muschietti *et al.* 1998; Kim *et al.* 2002). It has been proposed that LePRK1 and LePRK2, two pollen-specific LRR-receptor kinases from tomato, associate in mature pollen membranes as part of a multimeric protein complex with LAT52 as an extracellular partner. In the presence of style extract, LePRK2 is dephosphorylated and both LePRK1 and LAT52 are released. It was therefore proposed that this dissociation of the complex is triggered by a pistil ligand and that it results in a signal to the pollen tube (Wenger *et al.* 2003). Several proteins have been identified to interact with the complex (LeSHY, LeSTIG; Tang *et al.* 2004; KPP; Kaothien *et al.* 2005) reinforcing the idea that LePRK1 and LePRK2 might interact with different pistil-derived ligands during pollen tube passage through the various regions of the style.

It is poorly understood how cells are able to perceive even shallow chemical gradients over their relatively small size (few micrometers). This is particularly intriguing in the case of small molecules some of which are actually taken up and affect cellular metabolism: sugars (Reger *et al*. 1992), nitric oxide (Prado *et al*. 2004) and boron (Robbertse *et al*. 1990). Pollen tubes from many species also exhibit oxytropism *in vitro*, some positive and some negative (Blasiak *et al*. 2001). Similarly, certain ions elicit opposite responses in pollen tubes from different species.

Calcium was first identified as potential attractant in the snapdragon (*Antirrhunum majus*) pistil (Mascarenhas and Machlis 1962). It elicits chemotropic response in some species (*Pennisetum glaucum*, *Narcissus pseudonarcissus*, *Clivia miniata*) but not in others (*Lilium*, *Zea*). In *Arabidopsis*, calcium is provided by the stigma papilla cells (Iwano *et al*. 2004). In wheat, a gradient of calcium in the ovary might lead the way to the egg apparatus (Chaubal and Reger 1990). Calcium is involved in a plethora of cellular functions in plant cells (Hepler 2005). It is able to gelate acidic pectin polymers and therefore causes a rigidification of the cell wall. On the other hand calcium uptake through plasma membrane located channels is responsible for the high cytosolic calcium concentration in the apex of the pollen tube which is likely to directly interfere with growth as will be evident below. These calcium channels have been proposed to be stretch-activated and/or voltage gated (Feijó *et al*. 1995; Malhó *et al*. 1995; Malhó and Trewavas 1996; Pierson *et al*. 1996; Dutta and Robinson 2004; Wang *et al*. 2004).

pH shifts in the external medium to higher values can arrest pollen tube growth (Fricker *et al*. 1997). Whether pollen tubes actually change their growth direction in response to a pH gradient is largely unknown. A change in proton concentration is likely to influence the pattern of proton fluxes across the pollen tube plasma membrane, thus changing the characteristic pattern of cytosolic pH (Feijó *et al*. 1999). The cytosol in the very apex of the tube is more acidic than the adjacent subapical area. It has been proposed that the pH is a spatial and possibly also temporal organizer of cellular growth (reviewed in Chebli and Geitmann 2007). This function could be realized either by influencing exocytosis and/ or endocytosis, or actin dynamics by way of controlling the activity of actin binding proteins (Feijó *et al*. 1999). Alternatively, the electrical field generated by intracellular proton fluxes might control the movement of secretory vesicles. The perception of proton gradients is therefore likely to be immediate as the protons directly affect the growth machinery of the tube.

Pollen tubes show tropic behavior upon application of an external electrical field (Nakamura *et al*. 1991; Nozue and Wada 1993). It has been proposed that a cellular response to a larger voltage gradient is due to the difference in the membrane potential that is set up by the field, and the consequences of that difference on passive calcium entry into the cathode- and anode-facing sides of the cells (Robinson 1985). This effect could be enhanced by voltage gated calcium channels. Alternatively, the electrical field could result in an asymmetric distribution of ion channels by electrophoresis in the plane of the plasma membrane as demonstrated for muscle cells (Poo and Robinson 1977). Either way, influence of the external electrical field on the cytosolic calcium distribution has been postulated to be the key switch for determining pollen tube directional growth. It remains puzzling, however, that pollen tubes from different species react differently upon application of an electrical field with some pollen tubes growing towards the anode (*Impatiens*, tomato, tobacco; Wulff 1935; Wang *et al*. 1989) and others towards the cathode (*Vinca*, *Narcissus*, *Camellia*; Marsh and Beams 1945; Zeijlemaker 1956; Nakamura *et al*. 1991). How the different fields could nevertheless be integrated by the cytosolic calcium signaling step remains to be investigated.

The reaction to mechanical or anatomical cues is termed thigmotropism. Pollen tubes are presumed to be able to react to this type of signal (reviewed in Heslop-Harrison 1987) but the understanding of the perception mechanism is vague at best. A contact with a mechanical obstacle or a

change in the stiffness or viscosity of the growth matrix is likely to cause a local deformation of the tip of the cell and therefore alter the tension in the plasma membrane. This could possibly activate membrane located stretch-activated channels resulting in changing the ion flux across the membrane and thus affecting the local concentration of the respective ion. All recent models of the machinery organizing pollen tube elongation assume the existence of mechanically sensitive, stretch-activated calcium channels (Derksen 1996; Feijó *et al*. 2001; Holdaway-Clarke and Hepler 2003) and their presence was eventually demonstrated (Dutta and Robinson 2004). While these channels are seemingly necessary for regular pollen tube growth, they could also be involved in the thigmotropic response. Therefore, a mechanical signal seems to converge to the same calcium-based signaling pathway that is used by many of the other types of signals.

Intracellular signal transduction

Following the perception of an external signaling cue, the signal must be transmitted within the cell in order to eventually result in a cellular response. A crucial role in pollen tube growth and most probably in the transduction of many of the signals listed above is played by calcium. Growing pollen tubes are characterized by a cytosolic calcium gradient with concentrations of 2-10 µM at the tip dropping down to 20-200 nM in the shank region starting few micrometers behind the tip (Obermeyer and Weisenseel 1991; Rathore *et al*. 1991; Miller *et al*. 1992; Malhó *et al*. 1994; Pierson *et al*. 1994; Franklin-Tong *et al*. 1997). The area of high calcium corresponds to the region where cell wall expansion and fusion of secretory vesicles take place. Furthermore, it was observed that the gradient can be modified causing the tube to redirect its growth activity towards the zone of higher cytosolic calcium (Malhó and Trewavas 1996). In a series of studies on *Agapanthus* pollen tubes Malhó *et al*. (Malhó *et al*. 1994, 1995; Malhó and Trewavas 1996) were able to show that reorientation of pollen tubes can be triggered by transient increases in the cytosolic calcium concentration in the apical region of the pollen tube tip. The authors artificially shifted the position of the apical calcium gradient to the side of the tube apex using localized release of caged calcium. This treatment allowed them to modify the directional growth of the pollen tube.

The cytosolic calcium concentration is affected by both ion influx through the plasma membrane and liberation of calcium from intracellular stores (Pierson *et al*. 1994; Malhó *et al*. 1995). Calcium influx occurs mainly at the hemisphere-shaped tip of the pollen tube (Malhó *et al*. 1994; Feijó *et al*. 1995; Malhó *et al*. 1995; Pierson *et al*. 1996; Holdaway-Clarke *et al*. 1997). High cytosolic calcium plays a role in vesicle transport, stimulates exocytosis (Picton and Steer 1985; Battey and Blackbourn 1993; Roy *et al*. 1999), and has been shown to disrupt the actin cytoskeleton in lily pollen tubes (Kohno and Shimmen 1987). Most of these effects are mediated by the binding of calcium to proteins whose activity is altered as a result. Calcium homeostasis is likely to involve calcium pumps, one of which has been found to be required for normal pollen tube growth and fertilization in *Arabidopsis thaliana* (Schiøtt *et al.* 2004). Interestingly, a high number of pollen tubes from knockout mutants of this autoinhibited calcium ATPase (*aka9*) fail to discharge the sperm once in contact with the embryo sac resulting in an abortion of the fertilization process. It is not quite clear whether this has to do with a role of calcium in intracellular signaling or with any of the other functions of the ion.

Several calcium-triggered second messenger systems have been suggested to be downstream of calcium in the intracellular signaling pathways (**Fig. 4**). A primary decoding process of calcium information acts via calmodulin which in turn influences the activities of numerous enzymatic, cytoskeletal and structural proteins (Vogel 1994). Calmodulin is a calcium binding protein that can bind 0, 2 or 4 calcium ions and in each state it binds and regulates different proteins. Its intracellular distribution in pollen tubes appears to be uniform (Moutinho *et al*. 1998a), but its

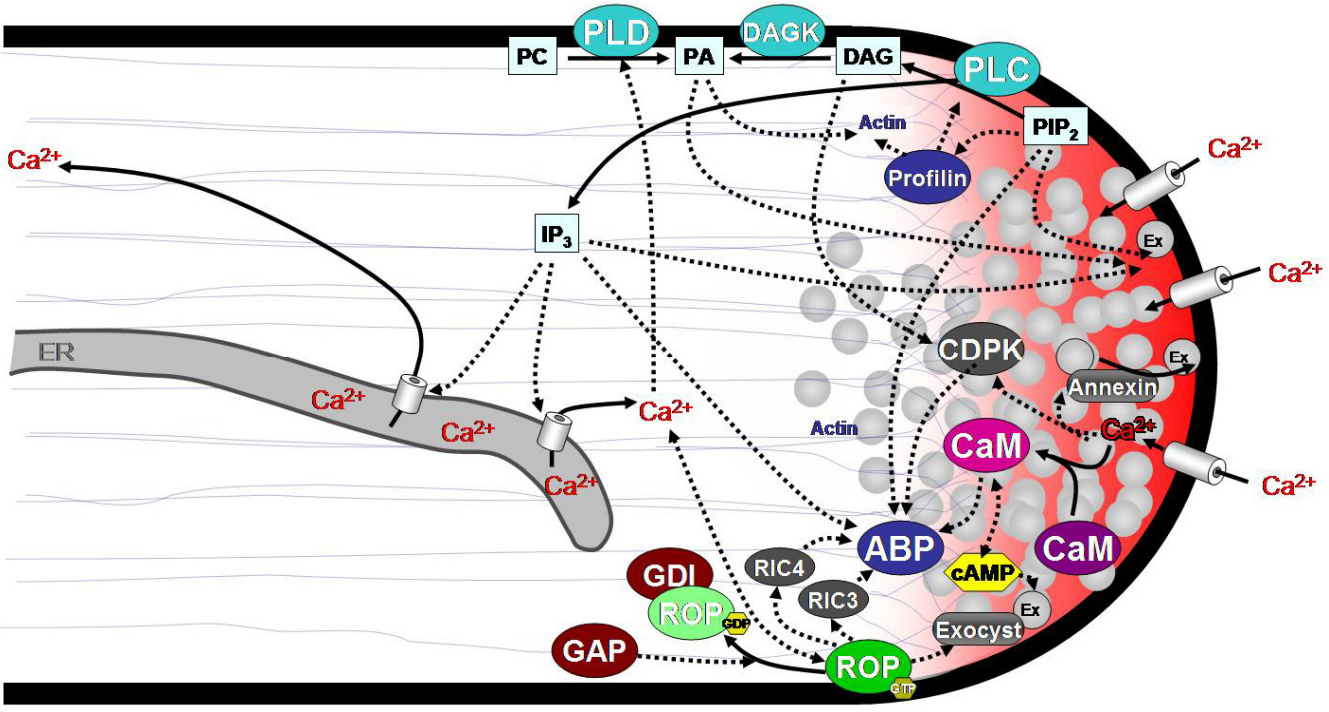

Fig. 4 Schematic model illustrating the main signal transduction pathways identified or proposed in pollen tubes. Solid arrows indicate molecular translocations or transformations. Dotted arrows indicate the direction of influence, but do not distinguish between positive and negative effects. Each arrow might represent multiple steps. Shades of red in the cytoplasm indicate the relative level of calcium concentration. ABP - actin binding proteins, CaM - calmodulin, DAG - diacylglycerol, DAGK - diacylglycerol kinase, ER - endoplasmic reticulum, Ex - exocytosis event, GAP - RhoGTPase activating protein, GDI - Rho guanine nucleotide dissociation inhibitor, IP_3 - inositol 1,4,5-trisphosphate, PA - phosphatidic acid, PC - phosphatidyl choline, PIP_2 -phophatidylinositol 4,5-bisphosphate, PLC - phospholipase C, PLD - phospholipase D, ROP - Rop/Rac GTPases.

activity exhibits a tip-focused gradient, similar to that of cytosolic calcium (Rato *et al*. 2004). Calmodulin has numerous targets such as inositol 1,4,5-triphosphate receptors and Ca^{2+}-ATPases, phosphodiesterase, myosin light-chain kinase and other protein kinases. In pollen tubes an artificial decrease in calmodulin levels in one side of the apical dome led to growth axis reorientation to the opposite side confirming the involvement of calmodulin in the transduction of directional signals (Rato *et al*. 2004).

Another major player in intracellular signaling is the family of phosphoinositides. A central molecule is phophatidylinositol 4,5-bisphosphate (PIP_2), the accumulation of which in the pollen tube apex has been proposed to play a regulatory role through the influence on membrane dynamics or cytoskeletal structure (Dowd *et al*. 2006). PIP_2 can be hydrolysed into two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C. A gene for phospholipase C (PLC) from *Petunia inflata* pollen (PetPLC1) has been cloned and the protein localizes to the apical region when fused to GFP (Dowd *et al*. 2006). In *Nicotiana tabacum* on the other hand NtPLC3 has been located at the flanks of the tube tip and it was proposed that this position limits the lateral spreading of PIP_2 (Helling *et al.* 2006). Despite the high sequence conservation between PetPLC1 and NtPLC3, the two proteins do not seem to be functionally identical.

Although PIP_2 localization seems to affect tip growth directly, it can also impact tip growth indirectly through its metabolites. Both cleavage products of the PLC reaction modulate the activity of downstream proteins during cellular signaling. IP_3 is able to stimulate calcium release from intracellular stores (Franklin-Tong *et al*. 1996; Malhó 1998). It is thus able to transduce signals from the tip to other regions of the cell and to amplify the initial stimulus-induced calcium signals. Depending on the local calcium concentration the IP_3 receptor reacts differently: In the apex, where the calcium concentration is elevated, the receptor undergoes an intrinsic inactivation upon IP_3 binding. In the shank region, where the calcium concentration is low, small increases in the cytosolic calcium concentrations potentiate calcium release from internal stores triggered by IP_3 (Dawson 1997).

The orientation of pollen tube growth can be modified by changing the intracellular concentration of both PIP_2 and IP_3. This has been achieved experimentally by the localized photorelease of these molecules from caged probes (Monteiro *et al*. 2005). Both molecules caused an increase of cytosolic calcium and growth perturbation. Interestingly, the two phosphoinositides seem to have different cellular targets, as the spatial distribution of the calcium signal was different and PIP_2 caused inhibition of apical secretion whereas IP_3 resulted in a stimulation of exocytosis. This points at the importance of calcium signatures – the precise spatial and temporal configuration of the calcium signal – for the transduction of signals (Rudd and Franklin-Tong 2001). Calcium signatures play also an important role in the transduction of signals involved in the self-incompatibility reaction, which will not be discussed here. Important work has been done on *Papaver rhoeas*, the field poppy, and for reviews on this topic the reader is referred to Rudd and Franklin-Tong (2001, 2003).

DAG has been proposed to be internalized at the flanks of the tube and recycled to the apex (Helling *et al.* 2006) but it can also be converted to phosphatidic acid (PA) through diacylglycerol kinase. The PA level is equally influenced by the activity of phospholipase D (PLD) which converts phophatidyl choline, phosphatidylethanolamine or phosphatidylglycerol into PA. This step is required for tip growth as has been shown by Potocký *et al*. (2003). It has been suggested that PA promotes the formation of secretory vesicles (Sweeney *et al*. 2002; Kooijman *et al*. 2003) and it might participate in the correct anchoring and positioning of actin filaments (Monteiro *et al*. 2005). In plants various PLD genes have been identified. The proteins they code for are regulated by calcium and G proteins (Zheng *et al*. 2000; Munnik 2001). Inhibitors of PLC and PLD activity result in abandoning anisotropic pollen tube elongation (Monteiro *et al*. 2005). By being targets for the phosphoinositide pathway, actin binding proteins provide a link between signal transduction and the cellular response which will be discussed below.

Protein kinases and phosphatases play key roles in signal transduction pathways as phosphorylation cascades are implied in major signal transduction routes. Numerous kinases are known to be expressed in pollen, many of them associated with the self-incompatibility reaction (for reviews consult McCubbin and Kao 2000; Rudd and Franklin-Tong 2001; Kachroo *et al*. 2002). Many of the plant kinases that have been identified are members of the superfamily composing the calcium-dependent protein kinases or calmodulin-like domain protein kinases (CDPK). Antisense suppression of pollen-specific CDPK in *Zea mays* impairs pollen germination and tube growth, as does addition of kinase inhibitors and calmodulin antagonists (Estruch *et al*. 1994). While protein kinases were shown to be distributed uniformly in the cytoplasm, their activity exhibits a tip-high gradient in *Agapanthus umbellatus* pollen tubes (Moutinho *et al*. 1998b). Reorientation of the pollen tube growth direction is accompanied by an asymmetric kinase activity within the pollen tube apex thus corroborating the hypothesis that a CDPK isoform acts as a downstream element in calcium signaling involved in the directionality of tip growth. A homolog of this *Agapanthus* CDPK isoform has been identified in *Petunia inflata* where overexpression of the gene causes tip bulging and is associated with elevated cytosolic calcium levels (Yoon *et al*. 2006). It might therefore be involved in calcium homeostasis. Another CDPK isoform from *Petunia inflata* is not involved in polarity but plays a role in pollen tube growth. Whether the main role of this protein for pollen tube growth is that of a kinase will have to be clarified (Yoon *et al*. 2006).

CDPKs have been proposed to be involved in the regulation of intracellular mechanical tension by affecting myosin and/or actin cross-linking proteins. Immunolabel of a CDPK in *Tradescantia* pollen tubes revealed a colocalization with F-actin thus providing support for the interaction of protein kinases with the cytoskeleton (Putnam-Evans *et al*. 1989).

The role of cyclic nucleotides in plant cell signaling is poorly understood. A cGMP transduction pathway seems to be involved in the transduction of NO signals (Prado *et al*. 2004). A putative adenylyl cyclase has been cloned in *Agapanthus umbellatus* pollen (Moutinho *et al*. 2001). Antisense silencing of the gene or treatment with antagonists of the protein resulted in disruption of pollen tube growth suggesting a requirement for cyclic AMP (cAMP) synthesis. The targets of the cAMP signaling pathway are still largely unknown. *In vitro* treatments of pollen tubes affecting cAMP levels in the cytoplasm resulted in transient elevations of the cytosolic calcium concentration. Furthermore, the local release of caged cAMP is able to cause a reorientation of tube growth, indicating that the cAMP interlinks with the calcium signaling pathway in regulating the directionality of pollen tube growth. Interestingly, inhibition of adenylyl cyclase resulted in a transient decrease in calmodulin activity. This suggests that downstream targets of cAMP are involved in the regulation of calmodulin, possibly through the cytosolic calcium concentration (Rato *et al*. 2004). There is, therefore, a link between the cAMP and the calmodulin signaling pathways.

Another group of central regulators of pollen tube growth are the Rop/Rac GTPases (Lin and Yang 1997; Kost *et al*. 1999; Li *et al*. 1999; Zheng and Yang 2000; Hwang and Zhang 2006). These small GTPases are a subset of the eukaryotic Rho class and act as molecular switches. They transduce signals in the GTP-bound conformation and return to an inactive GDP-bound state through GTP hydrolysis. Their critical role for tip growth has been shown by overexpressing the genes or expressing catalytically modified forms in *Arabidopsis* and tobacco pollen (Kost *et al*.

1999; Li *et al*. 1999; Fu *et al*. 2001). Overexpression of Rop/Rac results in the pollen tube abandoning growth anisotropy and assuming a sphere-shaped form. This phenomenon is exacerbated when Rop/Rac is constitutively active through irreversible binding of GTP (Li *et al*. 1999). Microinjection of Rop1Ps from garden pea in lily pollen tubes demonstrated that the protein interferes with the configuration of actin (Zhao and Ren 2006).

In yeast and animals the activity of Rho GTPases is controlled by GTPase-activating proteins (RhoGAPs), which stimulate GTP hydrolysis, and guanine nucleotide exchange factors (RhoGEFs), which catalyze GDP release to promote GTP binding. Another type of regulator are the guanine nucleotide dissociation inhibitors (RhoGDIs) which maintain the equilibrium between membrane-bound Rho GTPases with a cytoplasmic pool. A homolog of a RhoGDI, Nt-RhoGDI2, is co-expressed with the Rac/Rop GTPase Nt-Rac5 in tobacco (Klahre *et al*. 2006). The authors propose that Nt-RhoGDI2 mediates recycling of inactive, GDP-bound Nt-Rac5 from the flanks of the pollen tube to the cytoplasm, and that it is essential for the transport of this GTPase back to the apex, where it is reinserted into the plasma membrane and reactivated by nucleotide exchange. The inactivation of Nt-Rac5 was postulated to occur through the action of RhoGAP1, a GTPase activating protein that is located in the subapical region and thus is likely to confine the localization of the active GTPase to the apex (Klahre and Kost 2006).

The transduction of Rop/Rac activity occurs through multiple downstream effectors. Among these are Rop-interactive CRIB-containing proteins (RICs). Interestingly, two of these (RIC3 and RIC4) have been shown to play opposing roles in regulating actin dynamics (Gu *et al*. 2005). In tobacco, the loss of polarity induced by the overexpression of Rop/Rac GTPases can be rescued by a pollen-specific actin depolymerizing factor. The latter has therefore been proposed to be a downstream element in Rop/Rac signaling pathway (Chen *et al*. 2003). Rop/Rac GTPases have been associated with the control of vesicle fusion and endocytosis but this function is likely to be mediated by the actin cytoskeleton. Interestingly, Rop/Rac has also been found to act upstream of calcium in tip growth (Li *et al*. 1999). This might imply that this protein regulates the tip-localized influx of calcium and thus influencing the calcium gradient (Zheng *et al*. 2000). Furthermore, PIP_2 has been proposed to serve as Rop/Rac effector (Kost *et al*. 1999) and it might also be a positive regulator of NtRac5 (Helling *et al*. 2006) thus creating a positive feedback loop that helps polarizing Rac/Rob signaling and cell growth at the pollen tube tip. Another step that might mediate Rop signals is reversible protein tyrosine phophorylation, since inhibitors of this process interfere with pollen tube growth in a manner similar to overexpression of Rop (Zi *et al*. 2007).

Cellular response

The cellular responses by the pollen upon perception of a signal that are relevant for the *in planta* situation can be classified into three types: *a*) a change of growth rate causing acceleration, slowdown or arrest; *b*) a change of growth direction; *c*) lack or presence of adhesion (not discussed here, refer to Wilhelmi and Preuss 1996; Lord 2003). The most dynamic response is probably the signal-induced change of growth direction. To achieve this, the growth machinery of the pollen tube which is spatially confined to the very apex of the cell has to change position and shift towards the side of the apical dome. To comprehend how this shift of position can be achieved, it is important to understand the tip growth machinery (for a review see Chebli and Geitmann 2007).

The formation of a cylindrical cell is no trivial feat as turgor pressure, the driving force behind cell expansion, is an anisotropic force. To result in the highly anisotropic expansion typical for tip growth, the architectural features of the cell need to limit surface expansion to a small area. This is achieved by reinforcing the cell wall in the cylindrical part of the tube through the addition of cellulose and callose as well as through gelation of pectin polymers (Geitmann and Steer 2006; Chebli and Geitmann 2007). The growing apex on the other hand has a cell wall that consists almost exclusively of highly esterified pectins which are known to have a lower stiffness. This gradient in cell wall biochemistry along the longitudinal axis of the cell is reflected in the mechanical properties of the cell (Geitmann and Parre 2004). To change growth direction, the most pliable spot of the cell wall therefore has to shift. One way to achieve this is probably by redirecting the insertion of highly methyl-esterified and therefore "soft" pectin precursors to the new spot thus causing a cell wall softening at the new location. The location of vesicle fusion events thus needs to be redefined (**Fig. 5**). As mentioned earlier, signaling molecules such as PIP_2, IP_3, and PA are able to influence endo- and exocytosis. This occurs through a multiple pathway system, but how the spatial control of secretion events works mechanically is very poorly understood. The actin cytoskeleton is presumed to play an important role in this process. In root hairs it has been shown that a fringe like configuration of fine actin filaments confines the area where cellular expansion takes place (Ketelaar *et al*. 2003). The spatial control of actin polymerization and bundling activities is, therefore, likely to represent a key element in the cell's response.

Actin dynamics is controlled by numerous actin binding proteins which determine the rate of polymerization, depolymerization as well as that of filament bundling. Among the actin binding proteins identified in pollen are profilin, actin-depolymerizing factors (ADFs)/cofilins, gelsolins/vil-

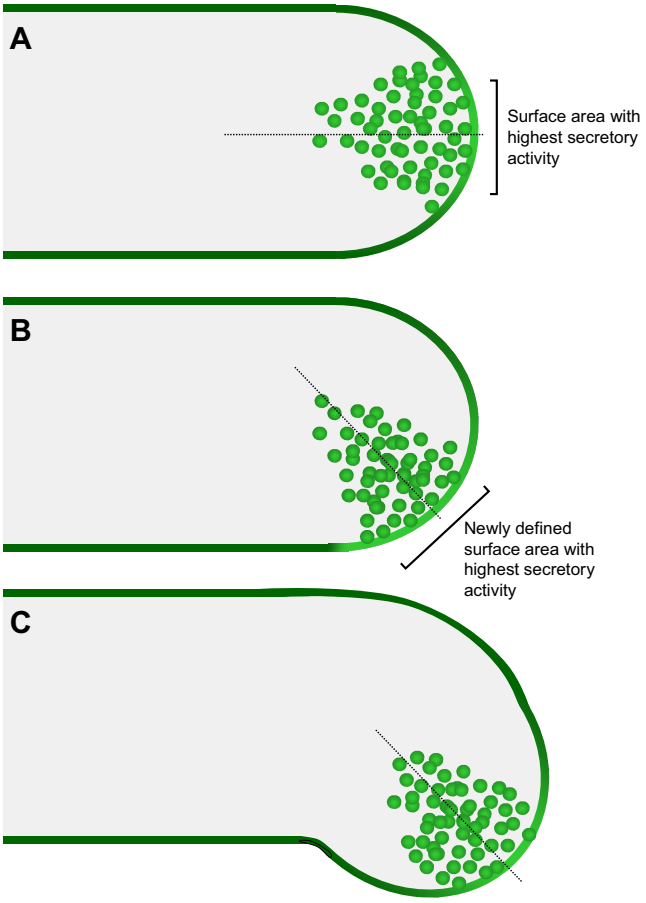

Fig. 5 Schematic representation of the processes that are likely to be involved in achieving a change in pollen tube growth direction. (**A**) The surface area with the highest rate of exocytosis is located on the central axis of the tube. (**B**) A change in localization of secretory events to a location positioned at the side of the tip results in a local softening of the cell wall. (**C**) The tube has changed its direction of growth. Shades of green in the cell wall indicate its degree of deformability.

lins, and formins (Staiger *et al*. 1997; Kovar *et al*. 2000; Fu *et al*. 2001; Hepler *et al*. 2001; Chen *et al*. 2002, 2003; Cheung and Wu 2004; Fan *et al*. 2004; Huang *et al*. 2004; Ren and Xiang 2007). Profilin is an actin-binding protein that forms complexes with ADP-actin promoting its phosphorylation to ATP-actin. It is abundant in pollen (Clarke *et al*. 1998) where it is uniformly distributed (Vidali and Hepler 1997). Depending on the conditions it can promote polymerization or depolymerization of filamentous actin. Interestingly, profilin can bind to PIP_2 thus regulating its levels and inhibiting the function of PLC (Drøbak *et al*. 1994).

Actin depolymerizing factors/cofilins are ubiquitous low molecular mass actin binding proteins important for regulating actin dynamics. They not only enhance actin depolymerization by binding preferentially to the minus, slow-growing end of actin filaments, but also can sever actin filaments. A pollen-specific actin depolymerization factor from tobacco (NtADF1) has been identified that associates with the subapical mesh of actin filaments at the pollen tube tip. It is important for maintaining normal pollen tube actin organization and overexpression leads to growth inhibition (Chen *et al*. 2002, 2003). Its activity may be spatially regulated by differential H^+ concentrations in the cytosol (Chen *et al*. 2002) and it was shown to be a downstream target of NtRac1, a tobacco Rac/Rop GTPase (Chen *et al*. 2003).

Gelsolins are calcium regulated actin binding proteins. When isolated from *Papaver rhoeas* pollen PrABP80, a gelsolin-like protein, severed actin and stimulated its depolymerization in a calcium dependent manner (Huang *et al*. 2004). However, it is also able to nucleate actin filaments. Formins are actin-nucleating proteins that stimulate the *de novo* polymerization of actin filaments. Overexpression of the *Arabidopsis* formin AFH1 in pollen tubes results in the formation of supernumerary actin cables as well as growth depolarization (Cheung and Wu 2004).

While the actin cytoskeleton probably represents the guiding rails along which the secretory vesicles are ushered to their target zone, the actual targeting of the vesicles to and the contact with the plasma membrane as well as the fusion events are mediated by a number of other proteins. These are likely to be involved in confining the fusion events to the apical secretion zone. Contrary to mammalian cells such as neurons, knowledge of the protein machinery controlling vesicle fusion in pollen tubes is fragmentary. A group of phospholipid-binding proteins which mediate exocytotic events is represented by the annexins. These proteins are able to bind to, aggregate, and fuse secretory vesicle membranes in a calcium-dependent manner. They were immunolocalized in the vesicle rich zone of *Lilium longiflorum* pollen tubes (Blackbourn *et al*. 1992) and are thus likely to be involved in the tip growth process, possibly by determining the spatial distribution of fusion events.

Another group of proteins involved in the targeting of exocytotic vesicles to the apical membrane are the Rab GTPases (Žárský and Cvrckova 1997). Rab11 localizes most prominently to the pollen tube apical region, the region that coincides with an accumulation of secretory vesicles. The expression of a constitutively active or a dominant negative variant of the protein in tobacco pollen resulted in reduced tube growth rate and meandering pollen tubes (de Graaf *et al*. 2005).

The exocyst is an eight-protein complex that is presumed to target and/or tether secretory vesicles at specific plasma membrane sites for exocytosis (Lipschutz and Mostov 2002). While orthologs of all exocyst components have been identified in *Arabidopsis* and rice, their roles in tip growth will yet have to be elucidated (Cole *et al*. 2005; Cole and Fowler 2006). Given that the exocyst has been shown to be regulated by a number of related GTPases in mammalian cells, it is an excellent candidate target for Rop regulation. Mutants of SEC8, one of the exocyst components, affect pollen tube growth or germination (Cole *et al*. 2005). Interestingly, the authors were able to determine that the germination impeding defect of the most severe mutant was based on the inability to form a pollen tube and not on a lack of the capacity to perceive the stigmatic signal initiating germination. More information on the exocyst function in the pollen tube will hopefully be available in the near future.

Knowledge is even more scarce for SNARE (soluble N-ethyl-maleimide-sensitive fusion protein attachment protein receptor) proteins. These protein complexes mediate the final stages of vesicle fusion throughout the endomembrane system and at the plasma membrane (reviewed by Sanderfoot and Raikhel 1999), but their presence and functionality in tip growth remain unexplored. A detailed understanding of the spatial control of exocytosis and growth in pollen tubes will be crucial for deciphering the mechanisms that allow pollen tubes to follow external guidance signals.

CONCLUSIONS

Sexual reproduction in flowering plants generates a very particular situation in which two genetically different organisms (the male gametophyte and the female sporophyte) get into intimate contact. The female sporophyte decides the fate of the male gametophyte by either permitting or preventing tube growth towards the ovule. In addition, the female sporophyte and gametophyte provide pollen tubes with guidance cues to find their targets. These signals need to be perceived, transmitted and translated into a cellular response. All of this requires continuous communication between the two partners; several components of these communication channels and the associated cellular machinery have been identified. However, to obtain the complete picture from the individual pieces of the puzzle will require additional work that represents a challenge for plant reproductive research.

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