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

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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.
Successful germination and growth of pollen 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), lyzes 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. 1997). In combination with pectin arabinogalactan protein, this 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 ary6 ary8 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 microple

After emerging from the transmitting tract, pollen tubes approach the ovule microple 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 in vivo pollen tube guidance have an extreme pollen tube guidance phenotype and include bel1 (Hülskamp et al. 1995), sin1 (Schnitz 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 diploid female sporophytic tube guidance phenotype and include 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 maat 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 microple. Based on these studies,
Emergence into ovary

**Funicular Guidance**

- *pop2* ino1

**Growth on funiculus**

- *pop2, ino1, myb98, anc1, ct1*

**Microplar entrance**

- *gfn2*

**Synergid cell degeneration**

- *feronia, sirene*

**Cessation of growth in synergid**

- *feronia, sirene*

**Tube burst**

- **Embryo sac**

- **Sperm Release**

**Double fertilization**

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

At the site of fertilization, and during later stages of pollen tube growth, the process termed pollen tube reception initiates after the pollen tube arrives at the female gametophyte but before pollen tube discharge (Sandaklie-Knikola et al. 2007). Evidence suggests that synergid cell degeneration is not essential for pollen tube reception, however. This conclusion is based on studies with *gfn2* 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 gametophyte 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 guidance; *mAa* mutants failed to enter the ovule (Sandaklie-Knikola 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 (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 the egg and central cell in vitro 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 super-numerary 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 defense, 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 *mAa* in *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 et al. 2003) 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...
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 (Reget 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 micropore (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 micropore (Palanivelu and Preuss 2006). In addition, repelled tubes stalled near the micropore (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 micropore 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 repel lent 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 so forth. However, in many instances interspecific hybridizations are not feasible due to incompatibility barriers, some of which occur during pollen tube growth and guidance on a pistil. For example, interspecific 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 an 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* trico 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 guidance system, if well designed, 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 invariant whether it grew through its own or through *A. arenosa*, *Olimarabidopsis pumilla* and *Sisymbrium* trico 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 of the target cell. The signals in the pollen tube are specific to the pollen tube. In tomato, the pollen tube is capable of responding to a variety of chemical cues, including thioglycoside, aliphatic thiols, and phenolic compounds. These signals are thought to influence the growth and development of the pollen tube, allowing it to navigate towards the female gametophyte.

In Arabidopsis, calcium is provided by the stigma papilla cells (Iwano et al. 2001; Potter and Beams 1999) and is thought to be involved in pollen tube guidance. Calcium entry into the pollen tube is thought to be mediated by voltage-gated calcium channels located in the plasma membrane (Hepler 2005). The influx of calcium into the pollen tube is thought to be regulated by the cytosolic calcium signaling step, which is thought to be the key to determining the growth and development of the pollen tube.

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 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 ovariain protein in Pennisetum glaucum (Renger et al. 1992). Other molecules that have been proposed to be involved in pollen tube guidance include γ-aminobutyric acid (Pananivela et al. 2003) and ZmEAI, 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 Arabidopsis LRR receptor kinase gene, MBL1, 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 receptors. In lily (Mollet et al. 2002) and tomato (Kim et al. 1998; Koo et al. 2002) has been proposed that LePRK1 and LePRK2, two pollen-specific LRR-receptor kinases from tomato, associate in the pollen tube. In Arabidopsis (Muschietti 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 Pennisetum glaucum (Renger et al. 1992). Other molecules that have been proposed to be involved in pollen tube guidance include γ-aminobutyric acid (Pananivela et al. 2003) and ZmEAI, 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 Arabidopsis LRR receptor kinase gene, MBL1, the effect on pollen tube growth is based on the enzymatic and therefore cytotoxic activity of the protein (Cruz-Garcia et al. 2003).

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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 (Schiott 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

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**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 - inositol 1,4,5-trisphosphate, PA - phosphatidic acid, PC - phosphatidyl choline, PIP2 - phosphatidylinositol 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-trisphosphate recep-
tors and Ca2+-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 trans-
duction of signals (Rato et al. 2004).

Another major player in intracellular signaling is the
family of phosphoinositides. A central molecule is phospha-
tidylinositol 4,5-bisphosphate (PIP2), the accumulation of
which in the pollen tube apex has been proposed to play a
regulatory role through the influence on membrane dyna-
mics or cytoskeletal structure (Dowd et al. 2006). PIP2 can
be hydrolysed into two second messengers, inositol 1,4,5-
trisphosphate (IP3) and diacylglycerol (DAG) by phospha-
lipase 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 NiPLC3
has been located at the flanks of the tube tip and it was
proposed that this position limits the lateral spreading of
the two phosphoinositides seem to have different cellular
targets, whereas IP3 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; 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 path-
way, actin binding proteins provide a link between signal
transduction and the cellular response which will be dis-

cussed below.

Protein kinases and phosphatases play key roles in sig-
nal transduction pathways as phosphorylation cascades are
implicated in major developmental and physiological reac-
tions. Besides calmodulin, many of the plant kinases known to be expressed in pollen, many of them
associated with the self-incompatibility reaction (for re-
views consult McCubbin and Kao 2000; Rudd and Frank-
lin-Tong 2001; Kachroo et al. 2002). Many of the plant
kinases that have been identified are members of the super-
family composing the calcium-dependent protein kinases or
calmodulin-like domain protein kinases (CDPKs). Antisense
suppression of pollen-specific CDPK in Zea mays impairs
pollen germination and tube growth, as does addition of ki-

nase 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 direc-
tion is accompanied by an asymmetric kinase activity within
the pollen tube apex. This correlation indicates 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 there-
fore 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 regu-
ation of intracellular mechanical tension by affecting
myosin and/or actin cross-linking proteins. Immunolabel of
a CDPK in Tradescantia pollen tubes revealed a colocaliza-
tion 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 Aga-
panthus umbellatus pollen (Moutinho et al. 2001). Anti-
sense silencing of the gene or treatment with antagonists of
the protein resulted in disruption of pollen tube growth sug-

gesting 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 eleva-
tions of the cytosolic calcium concentration. Furthermore,
the local release of caged cAMP is able to cause a reorienta-
tion of tube growth, indicating that the cAMP pathway is
involved in regulating the directionality of pollen tube growth.
Interestingly, inhibition of adenylyl cyclase resulted in a transient decrease in calmo-
dulin activity. This suggests that downstream targets of
cAMP are involved in the regulation of calmodulin, possi-
ibly 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 phosphatidic acid (PLD) with phospha-
tidylinositol 4-phosphate (PIP2) and phosphatidyl-
choline (PC) as substrates has been implicated in regulating the
tip growth of pollen tube growth is that of a kinase will
have to be clarified (Yoon et al. 2006).

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 influ-
enced by which isoform of phospholipase D (PLD) which work-
verts phosphatidyl 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 secreta-
ory 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;
Yoon et al. 2006). In

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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). Chemical inhibitors of 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-RhoGD12, is co-expressed with the Rac/Rop GTpase Nt-Rac5 in tobacco (Klahre et al. 2006). The authors propose that Nt-RhoGD12 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, PIP2 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 phosphorylation, 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 PIP2, IP3, 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 (ADF)/cofilins, gelsolins/vil...

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**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 actin filaments. Interestingly, profilin can bind to PIP, thus regulating its levels and inhibiting the function of PLC (Drobak 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 (NtADAF1) 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 GTAPase (Chen et al. 2003).

Gelsolins are calcium regulated actin binding proteins. When isolated from Papaver rhoesas 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 exocytic 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 exocytic vesicles to the apical membrane are the Rab GTAPases (Zárský and Cvrckova 1997). Rab1 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 exocytosis is an eight-protein complex that is presumed to target and/or tether secretory vesicles at specific plasma membrane sites for exocytosis (Lipschutz and Moscovich 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 GTAPases 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-ethylmaleimide-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 male 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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