

Gametophytic pollen tube guidance

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Abstract The concept of a pollen tube attractant was proposed in the late nineteenth century when pollen tubes were found to grow toward excised pistil tissues on medium. Since then, for about 140 years, plant biologists have tried to identify the pollen tube attractants. However, no molecule has been convincingly demonstrated to be the true attractant that actually controls the navigation of pollen tubes in the pistil. The past decade has seen substantial progress in this field in terms of our understanding of the various mechanisms of pollen tube guidance. It was suggested that diffusible pollen tube attractants might provide localized signals that affect the directional growth of the pollen tube, especially in the last phase of guidance by the target female gametophyte. Here, we review the mechanisms of pollen tube guidance, with special focus on the gametophytic guidance and the attractant. The necessary and appropriate conditions required by the true attractant will be discussed.

Keywords Pollen tube guidance · Attractant · Synergid cell

Introduction

Pollen tube guidance is the mechanism whereby the pistil tissue helps in navigating the pollen tube (male gametophyte) from the stigma to the target embryo sac (female gametophyte). The pollen tube grows inside the pistil without losing its way and finally arrives at the micropylar end of the embryo sac, where the surface diameter is narrow and close to that of the pollen tube. Chemoattractants for the pollen tube have been assumed to control such directional growth of the pollen tube in the pistil, and many plant biologists have tried to identify the attractants. In classical studies, biochemical and histochemical properties of putative attractants were reported using pistil tissues and their extracts from various species (Mascarenhas and Machlis 1962a; Reger et al. 1992), and one candidate, the calcium ion, was identified as the chemoattractant molecule (Mascarenhas and Machlis 1962b, 1964). There was an increasing gradient of the calcium concentration along the pistil, which was consistent with the classical hypotheses that a single attractant mediated the guidance from the stigma to the ovule. The external calcium ion is, however, a molecule necessary for the tip growth of the pollen tube, and it was argued that the assay systems could not discriminate between attraction and growth stimulation (Heslop-Harrison 1987). Since mechanical control by the architecture of the pistil tissue can also explain the pollen tube guidance, classical studies could not determine whether the attractant really existed.

What property is required for the presumed attractant? The presumed attractant may diffuse, form a gradient, and

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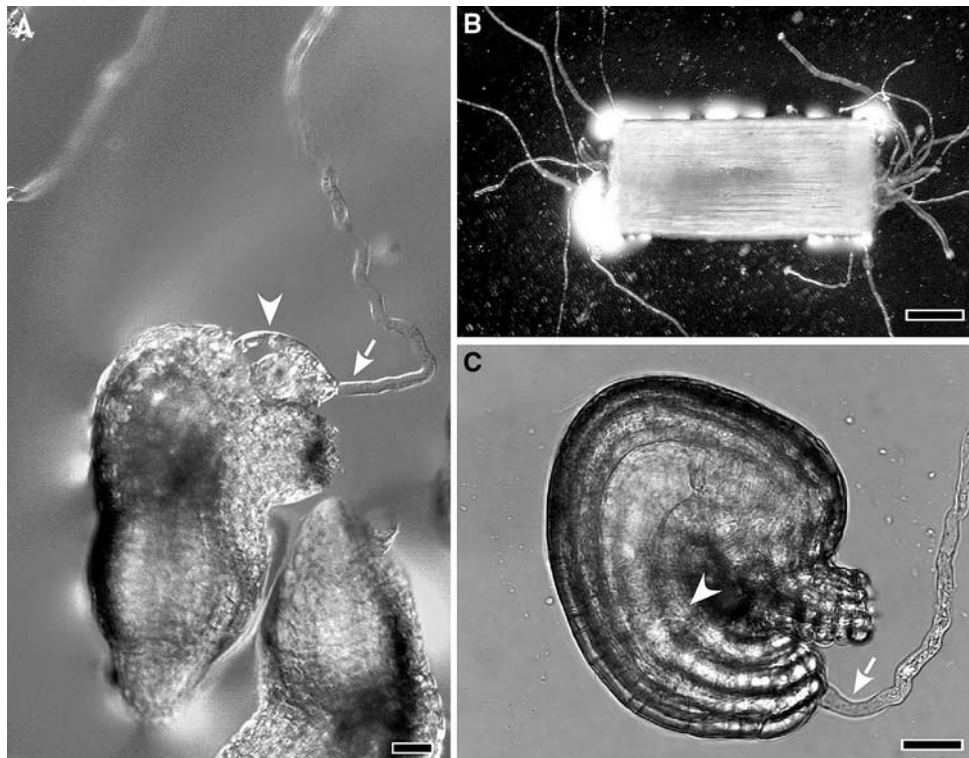


Fig. 1 Various in vitro systems. **a** In vitro *Torenia* system. A protruding embryo sac (*arrowhead*) provides ready access for viewing pollen tube reception (*arrow*) in *Torenia*. Such protruding embryo sacs facilitate manipulation of both embryo sac cells and pollen tubes (see text and Higashiyama et al. 1998, for details). **b** Dark-field image of pollen tubes of *Torenia* emerging from both ends of the cut style. Pollen grains were inserted into the middle of a cut style and cultured on the medium for the in vitro *Torenia* system.

Note that pollen tubes emerge not only from the correct end (the end close to the ovary) but also from the left side (the end close to the stigma) at similar frequencies. A similar experiment was originally performed in lily by Iwanami (1959). **c** An in vitro system for *Arabidopsis*. Pollen tube attraction to the micropyle of the ovule is shown, as described by Palanivelu and Preuss (2006). *Arrows* indicate attracted pollen tubes and *arrowheads* denote embryo sacs. Bar 20 μm in **a** and **c** and 500 μm in **b**

cause directional growth of the pollen tube toward the increasing gradient. It is now widely accepted that multiple stage-specific signals mediate pollen tube guidance (Johnson and Lord 2006). Search for attractant molecules in sporophytic tissues have identified several candidates of attractants, as well as molecules that perform other essential roles in pollen tube guidance, including growth stimulation and reorientation (Johnson and Lord 2006). In sporophytic tissues, presumed attractants do not trap pollen tubes at the highest concentration. Relation between a concentration gradient of each potential attractant and guidance still remains unclear.

Studies in this decade, however, have shown that such an attractant definitely exists in the last phase of pollen tube guidance by the female gametophyte (Higashiyama et al. 2003; Johnson and Lord 2006). Genetic studies using mutants of *Arabidopsis* defective in embryo sac development have shown that the female gametophyte governs the pollen tube guidance to the ovule (Hülkamp et al. 1995; Ray et al. 1997; Shimizu and Okada 2000). An in vitro system using *Torenia fournieri*, in which the embryo sac protrudes from the micropyle of the ovule, showed that the

female gametophyte clearly attracted the pollen tubes using a diffusible signal (Higashiyama et al. 1998; Fig. 1a). The two synergid cells proved to be the origin of the signal, as identified by a laser ablation experiment in the in vitro *Torenia* system (Higashiyama et al. 2001), and later by the *myb98* mutant of *Arabidopsis* (Kasahara et al. 2005). This attractant (or attractants) derived from the synergid cell is expected to be identified as the true attractant. Here, we review the mechanisms of gametophytic pollen tube guidance, with special focus on the attractant derived from the synergid cell. Characteristics and candidates for the attractant derived from the synergid cell will be discussed to provide understanding of the necessary and appropriate conditions required by the true attractant.

Mechanisms of gametophytic pollen tube guidance

Comparison to sporophytic guidance

Before entering the ovary, pollen tube guidance is governed by the sporophytic cells of the pistil. That pollen

tubes can grow through the stigma and style even in a pistil that lacks a female gametophyte (e.g., Hülskamp et al. 1995) has been demonstrated in a semi-in vitro system using a cut pistil in which the ovary has been removed (e.g., Higashiyama et al. 1998), or in plants in which megasporogenesis occurs after pollination (e.g., Sogo and Tobe 2005). These results suggest that the female gametophyte is not necessary for pollen tube guidance from the stigma to the base of the style in many species.

Not only chemotropic guidance but also mechanical guidance appears to be involved in the sporophytic guidance. For example, the lily style has been suggested to mechanically guide the pollen tubes because of its hollow architecture (Iwanami 1959). When pollen grains were placed onto the top end of a cut style, germinated pollen tubes emerged from the bottom end. In contrast, when pollen grains were placed at the bottom end of a cut style, germinated pollen tubes emerged from the top end of the style, growing in the “wrong” direction. When pollen grains were inserted into the middle of the stylar canal, germinated pollen tubes emerged from both ends of the style at equal frequency. The style of *Torenia* is also of the hollow type, and the same phenomenon occurs as found in the lily (Fig. 1b). These results suggest that no directional signal exists in lily or *Torenia* styles and pollen tubes growing straight emerge from the opposite end of the entrance, although the possibility cannot be excluded that this is due to limited or impaired competency of the pollen tube to receive the directional signal. In the tobacco style, which is of the solid type, glycosylated transmitting tissue-specific (TTS) proteins (arabinogalactan proteins; AGPs) are involved in pollen tube guidance (Cheung et al. 1995; Wu et al. 1995). Debate, however, is ongoing as to whether TTS proteins control the directional growth of the pollen tube or only sustain the elongation of the pollen tube (Higashiyama and Inatsugi 2006). Growth stimulation by the transmitting tissue was suggested in a *no transmitting tract (ntt)* mutant of *Arabidopsis*, which was defective in the development of the transmitting tract. Pollen tubes in the *ntt* pistil grew more slowly and were shorter, and fertilization efficiency was reduced in the lower half of the ovary (Crawford et al. 2007). Interestingly, pollen tubes at the top of ovary frequently grew toward the sides of the ovary chamber, suggesting that the transmitting tract of *Arabidopsis* is required for rapid apical-to-basal growth of the pollen tube in the pistil.

The stigma often appears to attract germinating pollen tubes in vitro. The lily stigma contains exudates and displays strong activity to attract (reorient) pollen tubes from lily species but not those of tobacco. Chemocyanin, a 9.9-kDa basic plantacyanin, was identified biochemically as the responsible molecule for chemotropism in lily (Kim et al. 2003), and chemocyanin protein was expressed most

abundantly in the stigma and style. The chemotropic activity of chemocyanin increased when combined with stigma–stylar cysteine-rich adhesin (SCA), and the chemocyanin fraction alone showed minimal activity at 0.23 $\mu\text{g}/\mu\text{l}$ and full activity at 0.69 $\mu\text{g}/\mu\text{l}$. However, neither binding activity with copper nor involvement in redox reactions has been shown for chemocyanin. The activity of recombinant chemocyanin expressed in other systems has not been reported.

Plantacyanin is a blue, copper cell wall protein that is often capable of redox reactions. In *Arabidopsis*, only one *plantacyanin* gene exists, which shows 51.9% identity and 86.8% similarity to lily chemocyanin at the amino acid level (Dong et al. 2005). *Plantacyanin* was expressed in various tissues, including the style of the pistil, and plantacyanin protein was most abundantly localized to the transmitting tract in the pistil tissues. Overexpression of *plantacyanin* caused aberrant growth of wild-type pollen tubes on the papilla cell in about half of the tubes. In these plants, the pollen tubes made several turns before growing into the style, but otherwise no differences were observed in pollen tubes emerging from the cut end of the style between the wild type and the overexpressor. No line has been found with a T-DNA insertion in the exon of *plantacyanin*, and the ability of purified *plantacyanin* of *Arabidopsis* to attract the pollen tube has not been demonstrated in vitro; thus, it is yet to be determined whether the plantacyanins of both lily and *Arabidopsis* actually govern the directional growth of the pollen tube in the pistil. It would be interesting to find whether a concentration gradient of plantacyanins is formed in the exudates of lily and in each papilla cell of *Arabidopsis*.

In contrast, on the stigma of tobacco with lipid-rich exudates, and on the dry stigma of *Arabidopsis*, an appropriate water gradient (flow) has also been suggested to be the directional cue (Lush et al. 1998; Wolters-Arts et al. 1998). Pollen grains of tobacco in unsaturated triacylglycerides directionally grew toward the water, but this did not occur if they were in saturated triacylglycerides. Such a water gradient might be a redundant signal available together with other chemoattractants on the stigma.

After entering the ovary, guidance of pollen tube requires the presence of a target female gametophyte, as described below, but sporophytic tissues may also contribute to the guidance in the ovary (reviewed in Johnson and Lord 2006). GABA is a candidate for the sporophytic guidance cue, and forms a concentration gradient in the pistil and is highest at the inner integument of the ovule (Palanivelu et al. 2003). *POLLEN–PISTIL INTERACTION 2 (POP2)* of *Arabidopsis* encodes a transaminase that degrades GABA. The GABA level increased several tens of times in the pistil of a *pop2* mutant, and the pollen tubes of the *pop2* mutant were sensitive to higher concentrations

of GABA. As a result, pollen tube guidance in the ovary was impaired when the *pop2* mutant was self-pollinated. Neither the ability of GABA to attract pollen tubes nor pollen tube guidance in a pistil with low level of GABA has been demonstrated.

Signaling in gametophytic guidance

Pollen tube guidance to the ovule is governed by the target female gametophyte in *Arabidopsis* (Hülkamp et al. 1995; Ray et al. 1997; Shimizu and Okada 2000) and in the in vitro *Torenia* system (Higashiyama et al. 1998). In TL1 of *Arabidopsis*, a heterozygote of the reciprocal translocation, half of the female gametophyte is genetically unbalanced to degenerate during ovule formation (Ray et al. 1997). Guidance by the female gametophyte can be genetically divided into two steps: from the surface of the septum to the funiculus, and from the entrance of the micropyle to the embryo sac. In *magatama* (*maa*) mutants, in which a delay occurred in the development of the female gametophyte, pollen tubes grew on the funiculus but lost their way at the entrance to the micropyle (Shimizu and Okada 2000). An interesting correlation between the embryo sac development and pollen tube guidance has also been reported in the intermittent pollen tube growth of alders (Sogo and Tobe 2005).

The mechanism of funicular guidance is unknown: some attractant might be emitted directly from the developing and mature female gametophyte, or some signal from the female gametophyte may evoke attraction in ovular sporophytic cells indirectly. Micropylar guidance, however, was suggested to be governed by the synergid cell. MYB98, a transcription factor specifically expressed in the synergid cell and trichomes of the leaves, was shown to be necessary for both the organization of the filiform apparatus of the synergid cell and micropylar pollen tube guidance (Kasahara et al. 2005; Punwani and Drews 2008, this issue). The synergid cell of *Arabidopsis* is likely to emit some diffusible signal because in an in vitro system, pollen tubes cultured on a medium are attracted to the micropyle of the ovule (Palanivelu and Preuss 2006; Fig. 1c), although the possibility of a contribution from the sporophytic cells along the micropyle, such as secretion of GABA (Palanivelu et al. 2003), cannot be excluded.

In the in vitro *Torenia* system, pollen tubes are directly attracted to the micropylar end of the protruding embryo sac, which the filiform apparatus of the synergid cells occupies (Higashiyama et al. 1998). Pollen tubes precisely control their direction of growth in the medium and need not contact either ovular sporophytic cells of the ovule or another region of the embryo sac (Fig. 2 and Movie S1). Once attracted, pollen tubes never leave the embryo sac

and often form narrow coils on the surface of the embryo sac before entering the sac. Moreover, when an ovule attracting a pollen tube is moved by micromanipulation, the pollen tube still moves toward the micropylar end of the embryo sac (Fig. 3 and Movie S2). Such behavior of pollen tubes suggests that some diffusible attractant is derived from the micropylar end of the embryo sac, and the tubes are trapped at the highest concentration of the attractant. The source of the attractant was identified by laser ablation as the two synergid cells (Higashiyama et al. 2001). Although the attractant is suggested to diffuse precisely from the filiform apparatus of the synergid cell (Figs. 2, 3, Movies S1 and S2), contribution of the ovular sporophytic cells to the pollen tube attraction cannot be excluded in the in vitro *Torenia* system. For example, another diffusible signal may be derived from the ovular sporophytic cells to attract the pollen tube into the range of precise attraction by the synergid cell.

After fertilization in *T. fournieri*, pollen tube attraction ceases, despite the persistence of one synergid cell (Higashiyama et al. 2001). Some active mechanism must halt the attraction, such as the attractant being no longer being secreted, degradation of the attractant, and/or repulsion of the additional pollen tubes. Nitric oxide (NO) was shown to work as a repellent of lily pollen tubes in vitro (Prado et al. 2004). In *Arabidopsis*, live imaging of wild-type ovules suggested that blocking the penetration of supernumerary pollen tubes occurs just after the first pollen tube enters the micropyle (Rotman et al. 2003; Palanivelu and Preuss 2006). The mechanism of blocking is impaired in *sirene* (*sir*; Rotman et al. 2003) and *feronia* (*fer*; Huck et al. 2003) mutants, which are defective in the same receptor-like kinase gene expressed in the synergid cell (Escobar-Restrepo et al. 2007).

The stigma/style tissue has been suggested as contributing to the competency of the pollen tube in response to the directional signal from the synergid cell of *Torenia* (Higashiyama et al. 1998) and *Arabidopsis* (Palanivelu and Preuss 2006). Such a function of the stigma/style tissue was partly retained by the stigma tissue (papillae) alone or diffusible molecules from the stigma/style tissue (Palanivelu and Preuss 2006), or by the style tissue alone (Higashiyama T, unpublished data), although the frequency of competent pollen tubes was reduced in such cases. The molecular mechanism underlying this phenomenon is unknown but of great interest.

The relationship between gametophytic pollen tube guidance and the development of the sperm cell in the pollen tube has been previously discussed (Lord and Russell 2002). In the in vitro *Torenia* system, this relationship is unclear because division of the generative cell always occurs before the emergence of pollen tubes from the cut end of the style (Higashiyama T., unpublished

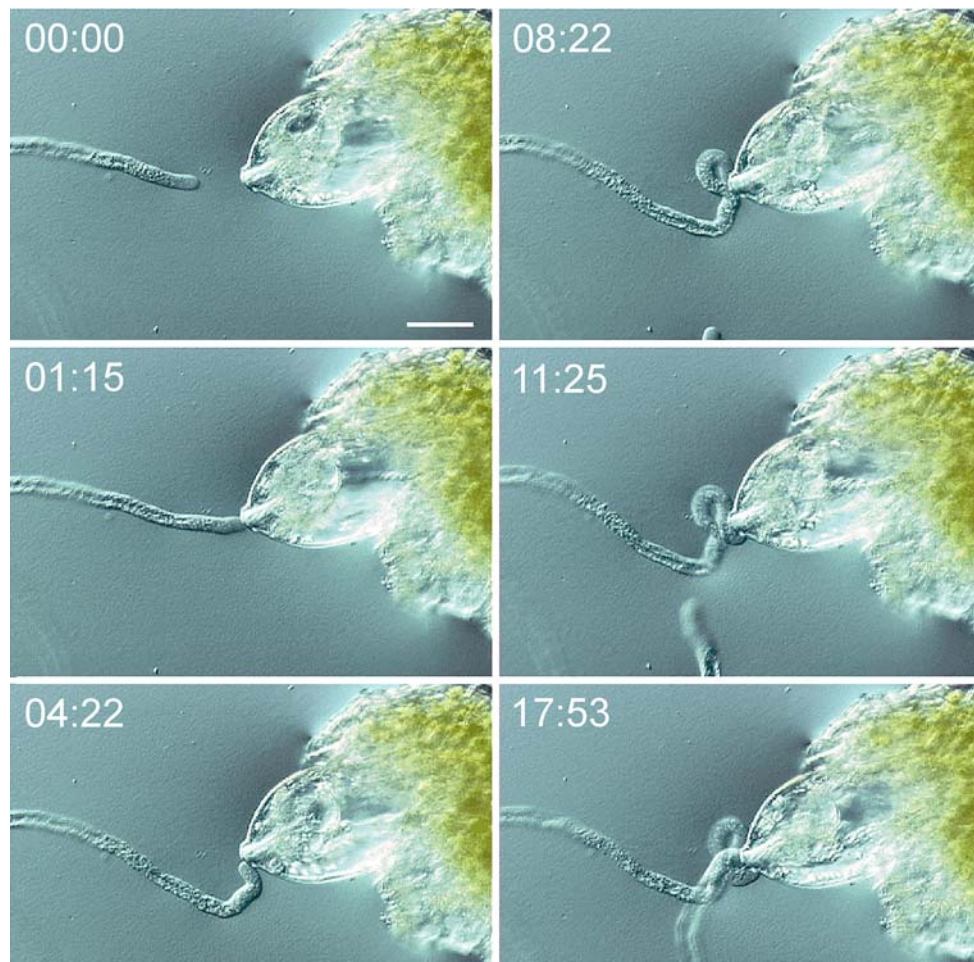


Fig. 2 Pollen tube attraction in the in vitro *Torenia* system. Two pollen tubes are attracted to the micropylar end of a protruding embryo sac. In the in vitro *Torenia* system, most pollen tubes do not enter the embryo sac smoothly, and often more than two pollen tubes

are attracted (Higashiyama et al. 1998). Blocking of supernumerary pollen tubes seems to occur after pollen tube discharge in this system. The movie file of this sequence is provided as a supplemental material (Movie S1). Time indicated in each panel is mm:ss. Bar 30 μ m

data). Neither pollen tube attraction at earlier stages nor inhibition of the division of the generative cell has been reported in *Torenia*. In *Arabidopsis*, the *cdka;1* mutant, defective in one of the cyclin-dependent kinases (CDKs), produces only one sperm cell (generative-like cell), but a defect in pollen tube guidance has not been reported (Nowack et al. 2006). The *duo1* mutant, defective in a *MYB* transcription factor required for sperm cell formation (Rotman et al. 2005), and the *generative cell specific 1* (*gcs1*) mutant, defective in a plasma membrane protein of the sperm cell required for fertilization (Mori et al. 2006), appear normal in pollen tube guidance. In contrast, the *hapless 2* (*hap2*) mutants, alleles of *GCSI*, showed impaired pollen tube guidance; in mutant pollen tubes, attraction to the female gametophyte decreased by about half, and abnormal growth path and growth arrest were observed in the ovary (von Besser et al. 2006). We await elucidation of the relationship between gametophytic

pollen tube guidance and the development of sperm cells. Fluorescent probes using *HISTONE3* (*H3*) variants, which first enabled us to perform live imaging of double-fertilization processes (Ingouff et al. 2007), would also be helpful such analysis.

The attractant(s) in gametophytic pollen tube guidance

Characteristics of the pollen tube attractant(s) derived from the synergid cell

The synergid cell is likely to attract the pollen tube in flowering plants, as judged from the similarities in structure and its function in accepting the pollen tube (Higashiyama 2002; Punwani and Drews 2008, this issue). However, involvement of the synergid cell in pollen tube attraction has been confirmed in *T. fournieri* (Higashiyama et al. 2001),

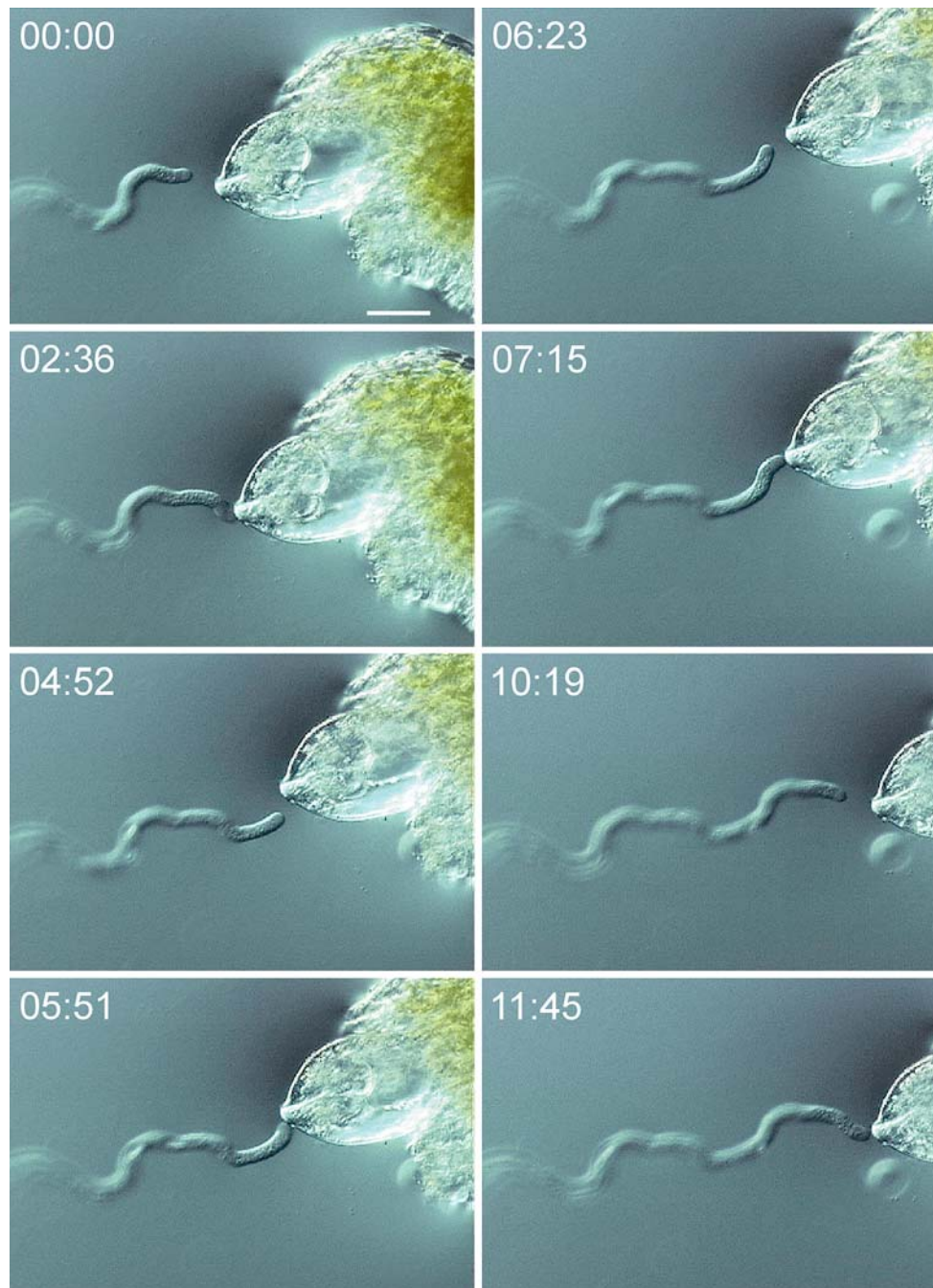


Fig. 3 Course of pollen tube elongation toward the embryo sac of a manipulated ovule. An ovule attracting a pollen tube in the in vitro *Torenia* system was inserted with a glass needle and manipulated with a micromanipulator. Note that the pollen tube is precisely following

the micropylar end of the embryo sac. The movie file of this sequence is provided as a supplemental material (Movie S2). Time indicated in each panel is mm:ss. Bar 30 μ m

Arabidopsis (Kasahara et al. 2005), and Scrophulariaceae species closely related to *T. fournieri*, including *T. baillonii*, *T. concolor*, *Lindernia crustacea*, and *L. micrantha* (Higashiyama et al. 2006). Here, we focus on the properties of the attractant derived from the synergid cell of these species.

The maximum distance of the attraction appears to be ~ 200 μ m in the in vitro *Torenia* system (Higashiyama

et al. 1998) and ~ 100 μ m (from the micropylar end of the synergid cell to the entrance of the micropyle) in *Arabidopsis* (Kasahara et al. 2005). In the in vitro system of *Arabidopsis*, pollen tubes that grew within ~ 100 μ m of an unfertilized ovule often made a sharp turn toward an ovule (Fig. 1c; Palanivelu and Preuss 2006). Such short-range attraction is consistent with the mathematical estimation

that 1.2–9.3 mm should be the maximum distance of attraction when a single, unbound molecule makes a concentration gradient and when a tube tip, 5–10 μm in diameter, can sense a 1–2% difference across the pollen tube (10^3 – 10^4 difference in total; Lush 1999). The chemoattractant protein for axon guidance, netrin, is effective over a distance of $\sim 300 \mu\text{m}$ in vitro (Kennedy et al. 1994). The attractant derived from the synergid cell is suggested to navigate pollen tubes in close proximity to the embryo sac, with a very high accuracy. In *T. fournieri*, two synergid cells attract more pollen tubes than a single synergid cell, implying that the distance of attraction is longer in the former case (Higashiyama et al. 2001). As estimated mathematically, the rate of production in the attractant might limit the maximum distance of attraction, especially when this rate is low (Goodhill 1997).

One characteristic of the attractant derived from the synergid cell is species preferentiality. The in vitro *Torenia* system is suitable for analyzing the species difference of the attractant because the attractant from the synergid cell spreads directly through the medium, and thus the possibility of sporophytic cells contributing along the path can be excluded. Plant species having a protruding embryo sac and suitable for cultivation, such as the in vitro *T. fournieri* system, were surveyed (Higashiyama et al. 2006). Plant species having protruding embryo sacs are phylogenetically divergent, suggesting parallel evolution. Some species of *Philadelphus*, *Thesium*, *Galium*, *Utricularia*, *Lindernia* (*Vandellia*), and *Torenia* have a protruding embryo sac (Maheshwari 1950). However, *Torenia* and the closely related genus *Lindernia* are suitable for cultivation, and *T. fournieri*, *T. baillonii*, *T. concolor*, *L. crustacea*, and *L. micrantha* have been used to examine species differences in the attractant. Laser ablation of both the synergid cells of these plant species stopped pollen tube attraction, suggesting their involvement in pollen tube attraction (Higashiyama et al. 2006).

When *T. fournieri* ovules were mixed with those of another species, and the pollen tubes grown, the pollen tubes preferentially tended to grow toward the embryo sac of their own species. In the most divergent combination, *T. fournieri* and *L. micrantha*, the pollen tubes grew specifically toward the embryo sac of their own species. Moreover, even when ovules were positioned with their embryo sacs facing toward the sacs of the other species, the pollen tubes still specifically grew toward the embryo sac of their own species, and interference between their respective attraction signals was not observed. These results suggest that the attraction is species-preferential, and that each species uses a different molecule(s) (not an order-specific attractant; Higashiyama et al. 2006). Such species preferentiality likely contributes to the reproductive barrier during in vivo crossing (Higashiyama et al. 2006). The frequencies of targeting the ovules of

Arabidopsis consistently decrease as the target plant species diverges in the in vitro system (Palanivelu and Preuss 2006), as well as in the in vivo crossing (Shimizu and Okada 2000; Hall et al. 2002). Interestingly, pollen tubes of *Mimulus hybridus*, which is a Scrophulariaceae species that has diverged considerably from *T. fournieri* (Wolfe and dePamphilis 1998; Albach et al. 2005), could arrive at the embryo sac of *T. fournieri* when crossed with the pistil of *T. fournieri*, suggesting that the pollen tube of *M. hybridus* can sense the attractant of *T. fournieri* (Kikuchi et al. 2007). A genome project for *Mimulus guttatus* is now in progress (<http://www.mimulusevolution.org/>), which may help to shed light on this phenomenon.

These pollen tube attraction results appear to be inconsistent with the hypothesis that the calcium ion might be the attractant of the pollen tube, or more precisely, the attractant derived from the synergid cell (reviewed in Higashiyama 2002). When the concentration of calcium in the medium was increased to 20 mM in the in vitro *Torenia* system, which is the maximum concentration that supports pollen tube growth, pollen tube attraction by the synergid cell still occurred (Higashiyama et al. 2006). Thus, the calcium ion may not be the sole attractant derived from the synergid cell. The calcium ion plays multiple roles during plant fertilization (Dumas and Gaudé 2006; Hepler et al. 2006). High concentrations of calcium in the synergid cell might be required for pollen tube–synergid cell interactions and/or fertilization processes (Higashiyama 2002; Punwani and Drews 2008, this issue). Species preferentiality observed in closely related species suggests that the attractant molecule has evolved rapidly. It is likely that the attractant is a molecule synthesized in the synergid cell, such as a peptide or protein. Lower plants, including algae, sometimes use hydrocarbons as sex pheromones, which are not specific at the species or genus level and mislead gametes of other species (Sekimoto 2005).

Other properties of the attractant derived from the synergid cells are that the secretion appears to be developmentally regulated in *Arabidopsis* (Palanivelu and Preuss 2006) and *Torenia* (Higashiyama T., unpublished data) and the onset of attraction is correlated with the development of the synergid cell. Heat-killing ovules stops the attraction in both *Torenia* (Higashiyama et al. 1998) and *Arabidopsis* (Palanivelu and Preuss 2006), but it has not been determined whether the attractant is heat-labile or if active secretion is necessary to maintain the gradient of the attractant. The molecular size of the attractant is unknown, but Palanivelu and Preuss (2006) estimated it to be $<85 \text{ kDa}$ based on diffusion rates of fluorescein-conjugated dextrans.

It is unknown whether the attractant derived from the synergid cell is a singular substance or a number of substances working redundantly or in a complex. No mutant

specifically defective in synthesis of a single attractant has been reported, implying the possibility that attractant substances might be redundant. On the other hand, reverse genetic analysis of gametophytic genes such as *MYB98* suggest that the forward genetic analysis has not been saturated yet.

Candidates for the pollen tube attractant(s) secreted from the synergid cell

Genes related to the attraction of pollen tubes that are expressed in the synergid cell have been investigated (Punwani and Drews 2008, this issue). *Zea mays EGG APPARATUS 1 (ZmEAI)*, which is expressed more abundantly in the synergid cells, is one candidate for being such an attractant, and was discovered using an EST analysis of a cDNA library of the egg cell (Márton et al. 2005). *ZmEAI*, a small protein of 94 amino acids predicted to be a plasma membrane protein, is the first identified female gametophytic protein related to pollen tube guidance. However, it is still unknown if purified *ZmEAI* has the ability to attract pollen tubes. Complex histochemical properties of the filiform apparatus and micropylar exudates (Huang and Russell 1992) imply the possibility that other molecules, not only the attractant, are secreted from the synergid cell.

ZmEAI was reported to be a member of the *EAI-like (EAL)* family, a large family of genes carrying the EA box (Gray-Mitsumune and Matton 2006). Five *EAL* genes were found to be expressed in various tissues of *Arabidopsis*, including the flower, but not specifically in the female gametophyte. Relation of *ZmEAI* with these *EAL* genes is discussed in this issue by Márton and Dresselhaus (2008).

Except for *ZmEAI*, no other candidate has been proposed as being the attractant derived from the synergid cell. Genes expressed in the synergid cell (summarized in Punwani and Drews 2008, this issue) as determined by an analysis of a cDNA library for the isolated female gametophytic cells (e.g., Márton et al. 2005; Yang et al. 2006), microarray analysis (e.g., Steffen et al. 2007), or reverse genetic analysis (e.g., Kasahara et al. 2005) might provide insights into not only the chemoattractant but also the molecular basis of pollen tube attraction by the synergid cell. Large-scale forward genetic analyses are also in progress (Johnson et al. 2004; Pagnussat et al. 2004).

The necessary and appropriate conditions for the true attractant

As discussed above, some molecule(s) synthesized in the synergid cell may be the true attractant and the guidance

cue that navigates the directional growth of the pollen tube in the ovary. The study on pollen tube attractants has a long history, but no true attractant has yet been identified. What are the necessary and appropriate conditions for the true attractant? One suggestion is that the candidate molecule, purified using some expression system or total synthesis, has the ability to attract the pollen tube, although it is difficult to demonstrate this ability convincingly. One difference between the in vitro *Torenia* system and classical in vitro systems is that the pollen tube is trapped at the highest concentration of the attractant in *Torenia* (Higashiyama and Inatsugi 2006; Fig. 2 and Movie S1). Pollen tubes can follow the source of the attractant precisely (Fig. 3 and Movie S2), and if one could demonstrate such an attraction using the purified molecule, it would be convincing. The other suggestion is that a specific deletion in the attractant molecule(s) makes pollen tubes lose their way. Even if a molecule has the ability to attract pollen tubes in vitro, it is necessary to show that the molecule is actually localized to the corresponding site and works as the guidance cue.

If these two conditions were clearly confirmed for a molecule, that molecule would be the true attractant. However, these two conditions have not been yet been met. For example, some antisense RNA and RNAi lines of *ZmEAI* are defective in pollen tube guidance (Márton et al. 2005), but the method used to attract pollen tubes is unknown. Chemocyanin has the ability to attract pollen tubes (Kim et al. 2003), but a molecular analysis for deletion or knockdown of chemocyanin in lily is still difficult. These analyses are hampered by difficulties associated with plant species but of great interest to be achieved. Search for other candidates are also continuing. As discussed above, analysis of genes expressed in the synergid cell is in progress, and the attractant derived from the synergid cell might be the peptide (protein) that rapidly evolves. We believe that these known candidates and/or novel molecules will be identified as the true attractant in the near future.

Conclusions

Substantial progress in this decade has elucidated several mechanisms involved in pollen tube guidance. Our next goal should be to identify the true attractant and its receptor, which has been a goal of plant scientists for more than a century. The properties of the attractant derived from the synergid cell have been characterized and a candidate, *ZmEAI*, has also been reported. Judging from the large species differences in the attractant, genes expressed in the synergid cell would provide insights into the molecular mechanism of pollen tube attraction. We expect the true attractant to soon be identified.

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