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Review

Pollen Tube Guidance by Attractant Molecules: LUREs

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ABSTRACT. **Sexual reproduction in flowering plants requires pollen-tube guidance, which is thought to be mediated by chemoattractants derived from target ovules. To date, however, no convincing evidence has been reported of a particular molecule being the true attractant. Emerging data indicate that two synergid cells, which are on either side of the egg cell, emit a diffusible, species-specific signal to attract the pollen tube at the last step of pollen-tube guidance. Recently, it was demonstrated that LUREs (LURE1 and LURE2), cysteine-rich polypeptides secreted from the synergid cell, are the key molecules in pollen-tube guidance. In this review, we summarize the mechanism of pollen-tube guidance, with special focus on gametophytic guidance and the attractants.**

Key words: pollen-tube guidance/attractants/synergid cell/peptide signaling

Introduction

During fertilization in flowering plants, the pollen tube (the male gametophyte) grows toward the embryo sac (the female gametophyte) in the pistil and delivers immotile male gametes to it (Fig. 1). As with axons in the developing nervous system, the directional growth of the pollen tube cell is controlled by complex interactions with the female reproductive system. Since the 1860s, many plant biologists expected that the ovule would emit some diffusible attractant(s). Early studies by Van Tieghem (1869) showed that pollen tubes grow toward excised ovules *in vitro*. In classical studies, the 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). One candidate, the calcium ion, was identified as a chemoattractant molecule (Mascarenhas and Machlis, 1962b, 1964). An increasing gradient of calcium concentration exists along the pistil, which is consistent with the classical hypothesis that a single attractant mediates guidance from the stigma to the ovule. However, since the external calcium ion is a molecule necessary for the tip growth of the pollen tube, it was argued that the assay systems used could not discriminate between attraction and growth stimulation (Heslop-Harrison, 1987). Moreover, a sugar molecule or an amino acid was also suggested as candidates. However, no convincing evidence of any specific molecule being the true attractant has ever been reported. The attractant proteins for axon guidance, the netrins, were successfully identified as secreted proteins of ~78 kDa that are partly homologous with laminin because neurons can only grow on medium when netrin is present (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Pollen tubes can grow in medium in the absence of an attractant. Since mechanical control by the architecture of the pistil tissue can also explain pollen-tube guidance, classical studies could not determine whether an attractant really existed.

Genetic experiments have demonstrated that pollen-tube guidance is a multiple-step process (Fig. 1), in which the early stages are controlled by sporophytic cells of the stigma, style, and transmitting tissue, and the final stages are controlled by the female gametophyte (Johnson *et al.*, 2004; Johnson and Lord, 2006). Studies in this decade have shown that such an attractant definitely exists in the final phase of pollen-tube guidance by the female gametophyte (Higashiyama *et al.*, 2003; Johnson and Lord, 2006). Genetic studies using mutants of *Arabidopsis thaliana* defective in embryo sac development have shown that the female gametophyte governs pollen-tube guidance to the

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Abbreviations: GABA, gamma-aminobutyric acid; CRP, cysteine-rich peptide/polypeptide; PCR, polymerase chain reaction; GFP, green fluorescent protein.

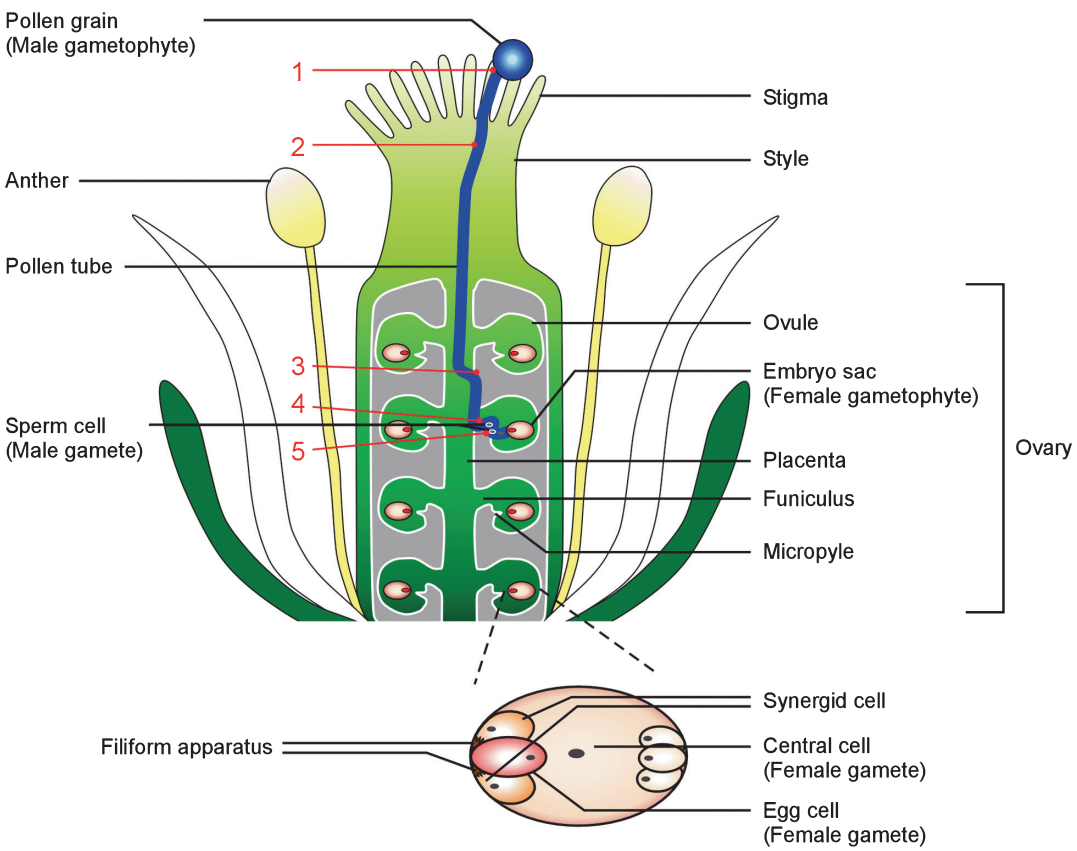

Fig. 1. Schematic representation of pollen-tube guidance. Pollen grain, the male gametophyte produced by anthers, reaches the stigma of the pistil. The pollen tube, which is a tip-growing cell, germinates from a hydrated pollen grain. The pollen tube elongates through the stigma and style to enter the ovary. The tube grows through the placenta and eventually arrives at the target embryo sac (female gametophyte) in the ovule. Pollen-tube guidance by the female gametophyte is critical for this step. Finally, two sperm cells (male gametes) within the pollen tube are discharged into the embryo sac through an interaction between the pollen tube and the embryo sac. One of these two sperm cells fuses with the egg cell to form the embryo and the other fuses with the central cell to form the endosperm via gametic interactions. Note that the central cell is not always defined as a female gamete since the fertilized central cell is not part of the next generation. In *Arabidopsis thaliana*, at least five steps are involved, as indicated with red numbers: stigma penetration (1), growth in the transmitting tract of the style (2), emergence from the transmitting tract of the placenta (3), funicular guidance (4), and micropylar guidance (5) (see details in Johnson and Lord, 2006).

ovule (Hülskamp *et al.*, 1995; Ray *et al.*, 1997; Shimizu and Okada, 2000). A semi-*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; Higashiyama and Hamamura, 2008). The two synergid cells, which are on either side of the egg cell, proved to be the origin of the signal, as identified by a laser ablation experiment in the semi-*in vitro Torenia* system (Higashiyama *et al.*, 2001), and later in the *myb98* mutant of *Arabidopsis* (Kasahara *et al.*, 2005). This attractant(s) derived from the synergid cell is thought to be the true attractant. Here, we review the mechanism of pollen-tube guidance, with special focus on attractants derived from the synergid cell.

Signaling in Sporophytic Guidance

Before the pollen tube enters the ovary, pollen-tube guidance is governed by the sporophytic cells of the pistil. Pollen tubes can grow through the stigma and style even in a pistil that lacks a female gametophyte (Hülskamp *et al.*, 1995). This has been demonstrated in a semi-*in vitro* system using a cut pistil from which the ovary was removed (Higashiyama *et al.*, 1998) and occurs in plants where megasporogenesis occurs after pollination (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 system. For example, the lily style has been suggested

to mechanically guide pollen tubes via its hollow architecture (Iwanami, 1959). When pollen grains were placed on 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, 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. These results suggest that no directional signal exists in lily styles, and that pollen tubes growing straight emerge from the opposite end of the entrance. However, 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, glycosylated transmitting tissue-specific (TTS) proteins (arabinogalactan proteins) are involved in pollen-tube guidance (Cheung *et al.*, 1995; Wu *et al.*, 1995). Debate is ongoing, however, 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 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).

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 molecule responsible for chemotropism in lily (Kim *et al.*, 2003). Chemocyanin protein is expressed most abundantly in the stigma and style. A fraction peak containing chemocyanin showed chemotropic activity at 0.23 μg/μl (~23 μM). Attraction activity was higher at 0.69 μg/μl (~69 μM) or when mixed with another protein that is a major compound in the active fraction of stigma proteins. This latter protein is a stigma/stylar cysteine-rich adhesin (SCA), which was identified as a lipid transfer protein involved in the adhesion of the pollen tube to the extracellular matrix of female tissues (Park *et al.*, 2000). SCA itself does not have chemotropic activity and might be an accessory protein important for the function of the chemocyanin (Kim *et al.*, 2003). The minimum concentration of chemocyanin necessary was estimated to be 0.05 μg/μl (~5 μM) in the presence of SCA (Kim *et al.*, 2003).

Plantacyanins are basic cell-wall proteins of unknown function, many of which are capable of redox reactions. Plantacyanins belong to a subfamily of blue copper proteins; however, one important amino acid for copper binding is substituted in chemocyanin, and whether chemocyanin can bind copper is not known (Kim *et al.*, 2003). 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* is expressed in various tissues, including the style of the pistil, and plantacyanin protein is most abundantly localized to the transmitting tract in pistil tissues. Overexpression of *plantacyanin* caused aberrant growth of wild-type pollen tubes on the stigma in about half of the tubes (Dong *et al.*, 2005). A T-DNA knockdown line, but not a knockout line, is available for the *plantacyanin* gene of *Arabidopsis*, but no phenotype has been observed. The ability of purified plantacyanin of *Arabidopsis* to attract the pollen tube has not been demonstrated *in vitro*. It has 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 out whether a concentration gradient of plantacyanins is formed in the exudates of lily and in the stigma of *Arabidopsis*.

After the pollen tube enters the ovary, its guidance requires the presence of a target female gametophyte, as described below, but sporophytic tissues may also contribute to guidance in the ovary (Johnson and Lord, 2006). GABA, gamma-aminobutyric acid, a candidate for the sporophytic guidance cue, 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 metabolizes GABA. The GABA level increases several ten-fold in the pistil of a *pop2* mutant, and the pollen tubes of the *pop2* mutant are sensitive to higher concentrations of GABA. As a result, pollen-tube guidance in the ovary is impaired when the *pop2* mutant is self-pollinated. Neither the ability of GABA to attract pollen tubes nor pollen-tube guidance in a pistil with low levels of GABA has been demonstrated.

Signaling in Gametophytic Guidance

Pollen-tube guidance to the ovule is governed by the target female gametophyte in *Arabidopsis* (Hülskamp *et al.*, 1995; Ray *et al.*, 1997; Shimizu and Okada, 2000) and in the semi-*in vitro Torenia* system (Higashiyama *et al.*, 1998). Guidance by the female gametophyte can be genetically divided into two steps. The first step is funicular guidance from the placenta to the funiculus of the ovule, and the second step is micropylar guidance from the entrance of the micropyle to the embryo sac. In *magatama* (*maa*) mutants, in which there is a delay in 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).

The exact mechanism of funicular guidance is unknown. It is conjectured that some attractant might be emitted directly from the developing and mature female gametophyte, or that some signal from the female gametophyte may evoke attraction in ovular sporophytic cells indirectly.

Micropylar guidance, however, has been suggested to be governed by the synergid cell. MYB98, a synergid cell-specific transcription factor, was shown to be necessary for both the organization of the filiform apparatus of the synergid cell, which is a cell-wall structure positioned as a gate, and micropylar pollen-tube guidance (Kasahara *et al.*, 2005; Punwani and Drews, 2008). The synergid cell of *Arabidopsis* is likely to emit some diffusible signal because, in an *in vitro* system, pollen tubes cultured on medium are attracted to the micropyle of the ovule (Palanivelu and Preuss, 2006). However, 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 semi-*in vitro Torenia* system, pollen tubes are directly attracted to the micropylar end of the protruding embryo sac (Higashiyama *et al.*, 1998). Pollen tubes specifically 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. Once attracted, pollen tubes do not leave the embryo sac, and often form narrow coils on the surface of the embryo sac before entering the sac. Moreover, when an embryo sac attracting a pollen tube is moved using a micromanipulator, the pollen tube specifically grew toward the micropylar end of the embryo sac (Higashiyama and Hamamura, 2008). This semi-*in vitro Torenia* system suggests that some diffusible signal is being derived from the micropylar end of the embryo sac. The source of the attractant was identified by laser ablation as being the two synergid cells (Higashiyama *et al.*, 2001). Although the attractant is suggested to be diffused specifically from the filiform apparatus of the synergid cell, the contribution of the ovular sporophytic cells to pollen-tube attraction cannot be excluded in the semi-*in vitro Torenia* system. For example, it is possible that 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.

The stigma/style tissue has been suggested to contribute to the ability of the pollen tube to respond to the directional signal from the synergid cell. In the semi-*in vitro Torenia* system, pollen tubes were required to grow through a cut style, suggesting that female sporophytic tissue promotes the ability of the pollen tubes to be attracted (Higashiyama *et al.*, 1998). Pollen tubes of *Arabidopsis* are also attracted to excised ovules more frequently when growing through a cut style (Palanivelu and Preuss, 2006). Classical assay systems did not use pollen tubes growing through a style, which might be one reason that pollen-tube attractants were not previously found.

Characteristics of the Pollen-Tube Attractant(s) Derived from the Synergid Cell

As described above, the synergid cell is likely to attract the pollen tube in flowering plants. The involvement of the synergid cell in pollen-tube attraction has been confirmed in *T. fournieri* (Higashiyama *et al.*, 2001), *Arabidopsis* (Kasahara *et al.*, 2005), and Scrophulariaceae, a species closely related to *T. fournieri*, including *T. baillonii*, *T. concolor*, *Lindernia crustacea*, and *L. micrantha*, which has a protruding embryo sac (Higashiyama *et al.*, 2006). Here, we focus on the properties of the attractant derived from the synergid cell of these species.

In the semi-*in vitro Torenia* system, the maximum distance of attraction appears to be ~200 μm (Higashiyama *et al.*, 1998). In *Arabidopsis*, the *MYB98* gene, which is involved in micropylar guidance, is likely to be effective over a distance of ~100 μm (Kasahara *et al.*, 2005). The chemoattractant protein for axon guidance, netrin, is effective over a distance of ~300 μm *in vitro* (Kennedy *et al.*, 1994). The attractant derived from the synergid cell is suggested to navigate pollen tubes into close proximity with the embryo sac, with very high accuracy. In *T. fournieri*, two synergid cells attract more pollen tubes than a single synergid cell, implying that the former case has a greater distance of attraction (Higashiyama *et al.*, 2001). The rate of production of 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 its species preference (Higashiyama *et al.*, 2006). *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, the growing 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 *in vivo* crossing (Shimizu and Okada, 2000; Hall *et al.*, 2002).

These pollen-tube attraction results appear to be inconsistent with the hypothesis that the calcium ion might be the pollen tube attractant, or more precisely, the attractant

derived from the synergid cell (Higashiyama, 2002). When the calcium concentration in the medium was increased to 20 mM in the semi-*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 Gaude, 2006; Hepler *et al.*, 2006). High concentrations of calcium in the synergid cell might be required for pollen tube-synergid cell interactions and/or the fertilization processes (Higashiyama, 2002; Punwani and Drews, 2008). 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.

Identification of Pollen-Tube Attractants Secreted from the Synergid Cell

Attractant molecule LUREs (LURE1 and LURE2) were identified by expressed sequence tag (EST) analysis of the synergid cell of *Torenia* (Okuda *et al.*, 2009). Synergid cells released by cell-wall degradation enzymes were collected under a microscope, and 25 synergid cells were used to establish a cDNA library via 30 PCR cycles. LUREs were the focus in the EST data, as the number of ESTs of LUREs was extremely high due to their abundant expression, which might be an important property for the attractant gene to retain a steep concentration gradient around the embryo sac. Moreover, LUREs are expressed specifically in the synergid cell. LURE proteins are secreted toward the micropylar end of the synergid cell and detected in the filiform apparatus, a characteristic cell-wall structure at the base of the two synergid cells.

LUREs are cysteine-rich polypeptides (CRPs) of ~9 kDa (~70 amino acids) with six cysteines, belonging to a subgroup of defensin-like proteins. Consistent with strict specificity, CRPs generally show rapid molecular evolution (Silverstein *et al.*, 2007). EST analysis of the synergid cell of *Torenia* revealed that various *CRP* genes (*TfCRP1–16*) are abundantly expressed in the synergid cell (Okuda *et al.*, 2009). Among the major three CRPs, TfCRP1 and TfCRP3 were identified as LURE1 and LURE2, respectively. Another gene, *TfCRP2*, was not shown to possess activity to attract pollen tubes. TfCRP2 is a smaller defensin-like protein (48 amino acids) with eight cysteines, predominantly expressed in the synergid cell among ovular cells like LUREs, but also expressed in anthers and developing fruits. Whether the remaining 13 TfCRPs (TfCRP4–16) are attractants is still not known. Downregulation of each LURE by morpholino antisense oligos decreases the frequency of pollen-tube attraction, suggesting that LUREs are involved in pollen-tube attraction by the synergid cell. Because genes for CRPs are known to rapidly diverge from each other (Silverstein *et al.*, 2007), it is difficult to find orthologs in other plants by using molecular phylogenetic analysis.

A bead method was developed to quantitatively investigate the activity of TfCRPs for pollen-tube attraction, whereby proteins were embedded in gelatin beads and placed in front of the pollen tube (~50-μm distance) by micromanipulation (Fig. 2A, 2B, and 2C). The beads, ~40 μm in diameter, gradually melted on the medium. Recombinant proteins of both LURE1 (TfCRP1) and LURE2 (TfCRP3) expressed in *E. coli* have strong activity to attract pollen tubes of their own species *in vitro* (Fig. 3A and 3B). Table 1 summarizes the characteristics of the attraction activities of LURE1, LURE2, TfCRP2, and the embryo sac (synergid cells) under different conditions. Neither non-competent pollen tubes nor pollen tubes of another species (*L. micrantha*) were attracted by these recombinant proteins (Fig. 3C). This result demonstrates that the activity of LUREs is consistent with that of the attraction signal derived from the synergid cells of *T. fournieri*. The tertiary structure of LUREs is likely to be stabilized by intramolecular disulfide bonds between the six cysteines and might be critical for pollen tube attraction, since a skip of the refolding process or heat treatment drastically reduces their activity. At optimum concentration, both LUREs attract about 60% of the pollen tubes, which is equivalent to the frequency of attraction by the synergid cell. The optimum con-

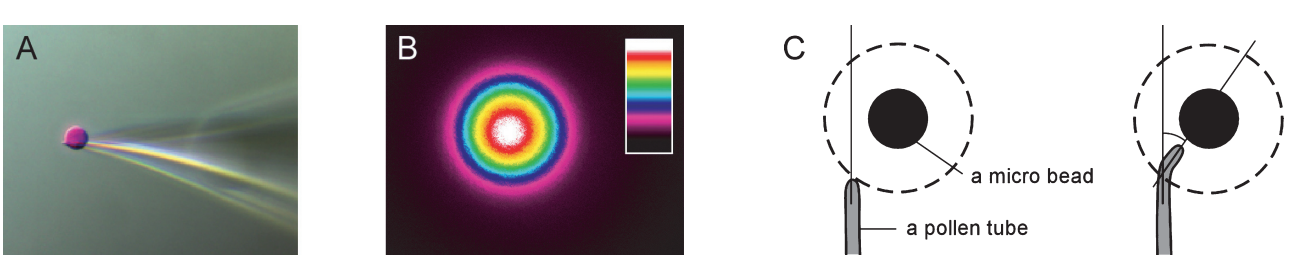

Fig. 2. The bead method for the *in vitro* attraction assay. A. The gelatin bead containing LURE1 or LURE2 mixed with 10 kDa Alexa Fluor was moved with a glass needle using a micromanipulator. B. Spectral colors correspond to the intensity of fluorescence (concentration of the Alexa Fluor dye), with white representing the highest level (see color scales). C. Schematic representation of the criteria for judgment of attraction. The 20° angle was determined by comparing the behavior of randomly meandering pollen tubes and pollen tubes growing toward beads containing LURE1 or LURE2 proteins.

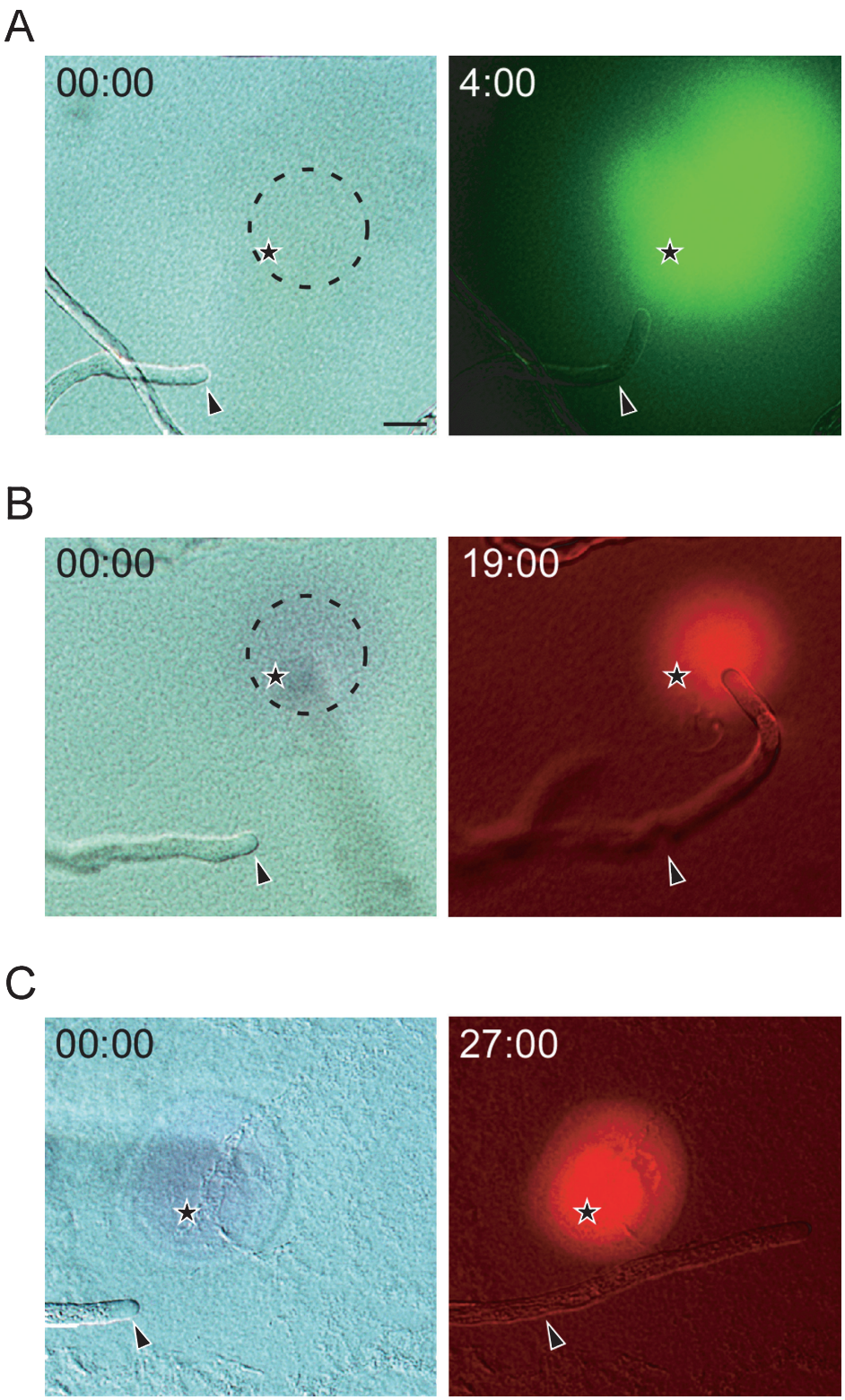

Fig. 3. Attraction of the pollen tube by recombinant LUREs. A, B. Pollen tubes growing through a cut style (competent pollen tube) were attracted by LURE1 (A) and LURE2 (B) mixed with 10 kDa Alexa Fluor. The gelatin bead (asterisk) containing LURE1 (A) or LURE2 (B) was placed in front of the pollen tube using a micromanipulator at time point of 00:00 (mm:ss). The tips of the tube (arrowhead) grew toward the bead (A, 4:00; B, 19:00). C. Pollen tubes germinated *in vitro* and grown on the medium (noncompetent pollen tubes) were not attracted by the bead containing LURE2. Green (A) or red (B, C) fluorescence shows the remaining concentration gradient of 10 kDa Alexa Fluor at 10 min increments after placement of the bead. Scale bar, 20 μm.

Table I. CHARACTERISTICS OF ATTRACTION ACTIVITIES OF RECOMBINANT PROTEINS AND THE EMBRYO SAC

	Pollen tube[c]	Attractant activity[d]
Tf embryo sac	Semi-*in vitro*, *Tf*	+++
Recombinant LURE1	Semi-*in vitro*, *Tf*	+++
Recombinant LURE2	Semi-*in vitro*, *Tf*	+++
Synthesized TfCRP2	Semi-*in vitro*, *Tf*	–
LURE1; heat treated[a]	Semi-*in vitro*, *Tf*	+
LURE2; heat treated[a]	Semi-*in vitro*, *Tf*	–
LURE2; without refolding[b]	Semi-*in vitro*, *Tf*	+
MOC control	Semi-*in vitro*, *Tf*	–
Tf embryo sac	*In vitro*, *Tf*	–
Recombinant LURE1	*In vitro*, *Tf*	–
Recombinant LURE2	*In vitro*, *Tf*	–
Tf embryo sac	Semi-*in vitro*, *Lm*	–
Recombinant LURE1	Semi-*in vitro*, *Lm*	–
Recombinant LURE2	Semi-*in vitro*, *Lm*	–

Torenia fournieri and *Lindernia micrantha* are abbreviated as *Tf* and *Lm*, respectively.

[a] Heat treatment, 95°C for 5 min

[b] A skip in refolding processes

[c] Semi-*in vitro* means that pollen tubes grew through a cut style to become competent for the attraction signal (Higashiyama *et al.*, 1998). *In vitro* means that pollen tubes germinated on the medium and did not grow through the style.

[d] +++, attracting about 60% of pollen tubes; +, attracting about 20% of pollen tubes; –, attracting ~10% of pollen tubes.

centrations in beads for LURE1 and 2 are 40 nM and 4 nM, respectively, although the ratios of appropriately refolded LUREs in the protein solution have yet to be determined. Unexpectedly, both LUREs show attraction activity at 40 pM per bead, which means that it should be sufficient to generate an attraction signal at only ~1,000 molecules per bead. This implies the possibility that single-molecule imaging of LUREs might be performed to reveal their action mechanism to the pollen tube.

Many CRPs of *Arabidopsis* are also suggested to be expressed in the synergid cell, as CRPs are downregulated in the ovule of a knockout mutant of the *MYB98* gene (Jones-Rhoades *et al.*, 2007; Punwani *et al.*, 2007). Using GFP fusion, some CRPs were shown to be secreted in the direction of the filiform apparatus (Punwani *et al.*, 2007). In EST analysis of the isolated egg cell (Cordts *et al.*, 2001) and embryo sac (Yang *et al.*, 2006) of maize, various CRPs were shown to be expressed specifically in the female gametophyte. Expression of various CRPs might be a characteristic of the synergid cell. LUREs might have evolved from antimicrobial peptides of the synergid cell located at the gateway of the embryo sac. Whether CRPs function as attractant molecules in other plant species is still unknown.

Conclusions and Perspectives

Two LUREs have been identified to date, and four other candidate TfCRPs have a similar cysteine arrangement (Okuda *et al.*, 2009). Both LUREs are defensin-like proteins, the genes of which form a large gene family, i.e., 323 genes in *Arabidopsis* and 93 genes in rice (Silverstein *et al.*, 2005, 2007). LUREs possess a γ-core and cysteine-stabilized αβ motifs that are conserved in antimicrobial peptides (Silverstein *et al.*, 2005; Yeaman and Yount, 2007). It would be of great interest to determine whether these peptides have antimicrobial activity since it would provide insights into the evolution of pollen-tube attractants. A candidate for the attractant derived from the synergid cell has been reported in maize. *Zea mays* EGG APPARATUS 1 (*ZmEA1*), a small membrane protein predominantly expressed in the synergid cell, is necessary for micropylar guidance (Márton *et al.*, 2005). *ZmEA1* fused with GFP was shown to diffuse from the egg apparatus as the ovule develops, suggesting that it may be further processed on the plasma membrane. *ZmEA1* has been proposed to be an attractant protein (Dresselhaus and Márton, 2009), although further studies are needed.

Multiple attractants may have two important functions: they might allow molecular evolution of each gene, while possible differences in function (e.g., reorientation and trapping) might generate an accurate guidance signal. Note also that the concentration gradient of LUREs is likely to be a critical factor for the directional signal. This is indirectly supported by the fact that pollen-tube attraction to the protruding embryo sac of *Torenia* can be observed in semisolid medium but not in liquid medium, and that the micro-bead assay is achieved using gelatin but not agarose. Extremely abundant expression of LUREs in the synergid cell might also be important in retaining the steep long-distance concentration gradient. Our next goal should be to reveal the differences between LUREs in function and identify receptor(s) for LUREs, since this would shed light on the molecular mechanism of pollen-tube attraction.

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