Female gametophyte-controlled pollen tube guidance

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Abstract
During the evolution of flowering plants, their sperm cells have lost mobility and are transported from the stigma to the female gametophyte via the pollen tube to achieve double fertilization. Pollen tube growth and guidance is largely governed by the maternal sporophytic tissues of the stigma, style and ovule. However, the last phase of the pollen tube path is under female gametophyte control and is expected to require extensive cell–cell communication events between both gametophytes. Until recently, little was known about the molecules produced by the female gametophyte that are involved in this process. In the present paper, we review the most recent development in this field and focus on the role of secreted candidate signalling ligands.

Introduction
Double fertilization, a unique and characteristic trait of flowering plants (angiosperms), is a very subtle and accurately regulated process. Owing to the fact that the female gametes (egg and central cell) are deeply embedded into the maternal tissues of the ovule and ovary respectively, they are inaccessible to the non-motile male gametes (sperm cells). Thus angiosperms have acquired a specialized form of sperm cell delivery and evolved a tricellular male gametophyte [pollen grain/PT (pollen tube)], consisting of two sperm cells enclosed by a large vegetative tube cell, which is able to grow over long distances to deliver its cargo [1]. After hydration and germination on the stigma surface, pollen grains form PTs that first penetrate the intercellular spaces between the papillar cells of the stigma, then grow towards and through the TT (transmission tract) of the style and ovary. In order to reach unfertilized ovules, PTs have to exit the TT in epidercots, emerge on the surface of the placenta and grow along the epidermis of the funiculus towards the micropylar entry of the ovule, finally arriving at the hidden FG (female gametophyte).

The mechanism that precisely directs the PT over such a long distance through the pistil towards the FG has been studied for more than a century. It has been shown that, before entering the ovary, PT guidance requires intensive communication with the surrounding sporophytic maternal tissues, and the presence of the FG seems not to be necessary for the largest part of the PT’s journey [2–4]. However, after exit from the TT tissue and emergence on the surface of the placenta, PT guidance is thought to be primarily under gametophytic control, although the maternal sporophytic tissues of the ovary might also contribute to guidance cues towards the ovary [5]. Excellent recent reviews offering updates in the mechanic and molecular processes of PT germination and sporophytic guidance have been published elsewhere [6–9] and we therefore restrict the present review to PT guidance governed by the FG.

Genetic dissection of gametophytic PT guidance
To understand the various mechanisms and components of the FG-controlled last phases of PT growth and guidance, several approaches have been performed in different plant species, especially in the Brassicaceae model plant Arabidopsis thaliana and the Scrophulariaceae species Torenia fournieri. Genetic approaches were applied in various sporophytic and gametophytic Arabidopsis mutants to determine whether and at which steps the PT path may be influenced by the FG. These studies have shown that PTs are not attracted to either immature or incompletely formed ovules or to ovules lacking FGS, and, as a consequence, did not approach the funiculus [2,10]. In contrast, in mutants displaying FGS with less severe or later developmental defects, PTs grew along the funiculus, but did not enter the micropyle [11]. Hence these results, together with those obtained from the experiments performed in the in vitro Torenia system [3], suggested that FG-controlled PT guidance begins when PTs exit the TT and grow towards the funiculus of each ovule. The last path of PT attraction controlled by the FG was therefore divided into at least two steps: (i) guidance from the surface of the placenta to the funiculus (funicular guidance), and (ii) guidance from the entrance of the micropyle to the FG or embryo sac (micropylar guidance) [6,11,12]. Since a funiculus is lacking in grasses (Poaceae), gametophytic PT attraction involves only the latter phase [7].

In order to reach the FG and to execute fertilization, PTs entering from the funiculus in Arabidopsis first have

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Key words: Arabidopsis, maize, micropyle, pollen tube guidance, signalling, synergid, torenia.
Abbreviations used: CRP, cysteine-rich protein; FG, female gametophyte; GABA, γ-aminobutyric acid; pap2, pollen-pistil interaction2; PT, pollen tube; TT, transmitting tract; ZmEA1, Zea mays EGG-APK40A0251

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to find their way to the micropylar opening formed by the outer integuments. In this channel-like structure, PTs can easily reach the naked micropylar region of the FG consisting of the egg cell and two glandular-like cells, the synergid cells [13,14]. In the case of T. fournieri, the other prime model plant for PT attraction studies, its naked FG is even more exposed, protruding through the micropylar opening of the integuments [3]. After arrival at the micropyle, one PT enters one of the two synergid cells (the receptive synergid) directly via the filiform apparatus, a thickened area of secondary cell wall material generated via plasma membrane invaginations of both synergid cells [15–17]. In the Poaceae, the FG is additionally embedded by a few layers of stack-like nucellus cells, representing an additional hurdle before PTs can finally enter the embryo sac (Figures 1A and 1B) [18,19]. Similar to the eudicot model plants described above, one PT in grasses first targets the filiform apparatus and then releases its contents explosively into the receptive synergid cell [14,20]. Second PTs were rarely observed in the micropyle of wild-type ovules of several plant species [18,21,22], indicating either that attractant(s) are no longer secreted, degraded or inactivated, or that additional PTs are actively repulsed.

**Signalling in gametophytic PT guidance**

A complex signalling network seems to be required for PT guidance by the FG. However, the molecular mechanism of funicular guidance still awaits to be discovered, and it is still unclear whether there is a diffusible signal emitted directly from the developing and mature FG or an indirect signal that causes a change in the ECM (extracellular matrix) of sporophytic cells [12]. On the basis of genetic, cell ablation and in vitro investigations with excised Arabidopsis and Torenia ovules, micropylar or short-range PT guidance appears to be governed by species-specific chemotactic signals secreted by the synergid cells of the FG. The maximum distance of attraction in vitro is in the range of approx. 200 μm in T. fournieri [6], whereas approx. 100 μm has been observed in Arabidopsis and maize [7,15,18,21]. Laser-assisted cell ablation experiments with Torenia ovules containing naked FGs have shown further that the synergid cells are necessary and sufficient to attract PTs and that neither the egg cell nor the central cell seems to be involved in this process and are capable of generating sufficient amounts of guidance molecule(s). However, at least in maize, the egg cell seems to contribute to the production of the attraction signal in addition to the synergid cells [18]. It has been shown that, in Arabidopsis, even the central cell might play a role in micropylar PT guidance [23], although it might be possible that the corresponding central cell guidance (CCG) gene encoding a potential transcriptional regulator might act rather indirectly and be required, for example, for FG maintenance or maturation. This hypothesis is supported by the observation that once the central cell was destroyed by laser cell ablation in Torenia ovules, most of the FGs were no longer able to maintain their structure and deteriorated.

Furthermore, it was observed that this resulted in an alteration of PT guidance [22].

Ca²⁺ has long been considered a potential attractant because it plays an important role for PT tip growth, and high concentrations have been detected in the synergid cells [24]. However, in vitro pollination assays using the in vitro Torenia system have indicated that it either does not function as an attractant at all or it belongs to an attractant cocktail of molecules [25]. A role in micropylar PT attraction was also proposed for the GABA (γ-aminobutyric acid) molecule, because it forms a gradient in the pistil with the highest concentration at the inner integument of the ovule [5]. PTs were sensitive to higher concentrations of GABA and guidance was impaired after self-pollination in the pollen-pistil interaction2 (pop2) Arabidopsis mutant. The POP2 gene encodes a transaminase that degrades GABA [5,26]. However, until now, a chemoattractant role for the GABA molecule has not been demonstrated, and PT guidance in a pistil with low GABA levels has not been shown. Thus it is more likely that gametophytic PT attraction and sperm release depends on species-specific factors rather than on general molecules such as Ca²⁺ or GABA.

The first gene encoding a candidate extracellular ligand involved in PT attraction controlled by the FG was identified in maize. Zea mays EGG APPARATUS1 (ZmEA1), which is specifically expressed in egg and synergids cells, encodes a polymorphic precursor protein shown to be secreted to the cell walls of micropylar nucellus cells. PTs arrived at the micropyle of ZmEA1-knockdown plants without penetrating the intercellular space of micropylar nucellus cells, suggesting a role for ZmEA1 in short-range gametophytic PT guidance [18] (Figures 1B and 1C). An N-terminally cleaved predicted mature ZmEA1 protein of 49 amino acids is capable of attracting maize PTs directly at a low concentration (<10 μM) in vitro (Figures 1D–1F). Moreover, Arabidopsis ovules expressing ZmEA1–GFP (green fluorescent protein) fusion protein driven by the synergid-cell-specific MYB98 promoter are capable of attracting maize PTs in vitro (M.-L. Márton, A. Fastner and T. Dresselhaus, unpublished work), indicating that it is possible to overcome wide crossing barriers combining genetic engineering with the tools currently being developed in plant reproduction research.

Recently, secreted CRPs (cysteine-rich proteins) named LUREs have been identified as the FG-derived attractants in T. fournieri [27]. These polymorphic proteins belong to a subgroup of the DEFL (defensin-like) gene superfamily of CRPs and are synthesized and secreted by the synergid cells. Their recombinant mature proteins have been shown to attract competent PTs in vitro in a species-specific manner. Moreover, microinjection of LURE antisense oligomers into mature embryo sacs resulted in a significantly impairment in PT attraction, providing additional evidence that LUREs 1 and 2 are the micropylar PT attractants derived from the synergid cells of T. fournieri.

A number of additional genes and proteins have been postulated to play a role in funicular and micropylar PT guidance in Arabidopsis [28–33]. However, the majority of the
Figure 1 | PT guidance by ZmEA1 signalling in maize

(A) Depiction of PT attraction by the FG and sperm discharge inside the receptive synergid cell (RSY). Visible in the PT (dark blue) is the nucleus of the vegetative tube cell. The two sperm cells (violet) are already released into the RSY. Outer and inner integuments are shown in pink and orange respectively, and nucellus cells are shown in yellow. The persistent synergid cell is shown in green, the egg cell in red, central cell in white and antipodals in grey. (B) and (C) Monitoring of PT growth and sperm cell discharge 24 h after pollination in ovules of wild-type (wt) plants and ZmEA1-RNAi (RNA interference) plants (EA1-RNAi) using transgenic ActinP::GUS (β-glucuronidase) maize pollen. Modified from [18] with permission from AAAS. (B) PT releases its contents into the wild-type receptive synergid cell. (C) PT grew normally at the surface of the inner integument, but did not enter the micropyle and continued growth at random directions in approx. 50% ovaries of ZmEA1-RNAi lines. The arrows label the micropylar opening of the ovule. (D–F) In vitro PT-guidance assay using an N-terminal cleaved predicted mature ZmEA1 protein of 49 amino acids. (D) ZmEA1 was released using a microcapillary at a 50 μm distance from an active maize PT. (E) At 22 min after application, PT tip growth was reoriented towards the region where the ZmEA1 droplet was placed (*). (F) Once the PT finally reached its target, it stopped growing. Abbreviations: AP, antipodals; CC, central cell; EA, egg apparatus; MC, microcapillary; PG, pollen grain; RSY, receptive synergid cell. Scale bars, 50 μm.

genes seem to be required for PT growth, and FG maturation thus plays a more indirect or no role for PT guidance. Various studies have clearly indicated that only fully mature FGs are capable of producing/secreting the micropylar attractants [16,21]. In magatama (maa) mutants, for example, which contain FGs exhibiting late developmental defects, PTs grew along the funiculus, but not inside the micropyle [11,32]. Mutations in an Arabidopsis synergid-expressed gene encoding the transcription factor MYB98 showed an immature and less organized filiform apparatus that, as a consequence, affected micropylar PT guidance [15].

Concluding remarks

Despite the fact that the PT path through the pistil towards the FG has been studied for more than a century, we are
only now beginning to understand the underlying molecular mechanisms preceding gamete fusion in flowering plants. The last few years have seen tremendous efforts in finding the molecules derived from the FG that are involved in PT guidance, but only ZmEA1 in maize and LURE1 and LURE2 in *T. fournieri* have been identified to date that accomplish the properties of a true attractant. However, owing to the development of the necessary tools and methods as well as the identification of the first FG-derived attractants as highly polymorphic small proteins, further progress in this field is expected soon, and the identification of the respective *Arabidopsis* attractants and probably other plant species seems to be close. A major task now represents the identification of the attractant-specific receptors at the PT tip surface as well as the associated signal transduction cascades responsible for reorient PT growth direction. It will be exciting to find out whether small GTPases and ion channels that play a key role in animal sperm chemotaxis[34,35] play a similar role in PT growth direction in plants, or whether plants have evolved different mechanisms.

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**References**


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