Review Paper

The pollen tube journey in the pistil and imaging the in vivo process by two-photon microscopy

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Abstract

The process of pollen germination and tube growth in the pistil involves a series of cell–cell interactions, some facilitating fertilization while others prohibiting pollen tube access to the female gametophyte, either because of incompatibility or as a result of mechanisms to avert polyspermy and to ensure reproductive success. Understanding pollen tube growth and guidance to the female gametophyte has long been a pursuit among plant biologists, and observations indicate that diverse strategies may be adopted by different plant species. Recent studies in Arabidopsis, maize, and Torenia fournieri suggest that low molecular weight secretory molecules probably play major roles in the short-range attraction of pollen tubes to the female gametophyte. The process of pollen tube growth in the pistil occurs beneath several cell layers so much of the information that conveys the intimate partnership between penetrating pollen tubes and the female tissues has come from fixed samples and observations of in vitro pollen tube growth responses to female factors. A unique glimpse of the in vivo pollen germination and tube growth process is provided here by intra-vital two-photon excitation (TPE) microscopy of pollinated Arabidopsis pistils that remained on intact plants. Further discoveries of critical factors of male or female origins and how they control the pollen tube growth and fertilization process will broaden our understanding of the common themes and diverse strategies that plants have evolved to ensure reproductive success. The advancement of imaging technology to monitor pollination and fertilization and the development of probes to monitor various aspects of the pollen tube growth process, including pollen intracellular dynamics, will allow us to superimpose details obtained from studying pollen tube growth in culture conditions to interpret and understand the in vivo events.

Key words: Fertilization, pollen–pistil interaction, pollen tube growth, pollen tube guidance, two-photon microscopy, vital imaging.

Introduction

Reproduction in flowering plants relies on accurate and timely delivery of sperm cells by the pollen tube (the male gametophyte) to the embryo sac (the female gametophyte) inside an ovule for fertilization (Fig. 1) (Lord and Russell, 2002; Boavida et al., 2005). The journey of the pollen tube begins with its emergence from the vegetative cell of the pollen grain after adhesion to the stigma. A strictly apical cell growth process maintains the pollen tube cytoplasm and its cargo, the sperm cells, in the most proximal region of the tube as it elongates through the female sporophytic tissues.
The pollen tube journey begins with pollen germination on the stigma, where pollen tubes emerge from pollen grain apertures close to the papillae and rapidly lead to a short pollen tube that grows relatively fast along or just beneath the papillae cell wall (Fig. 2B–D; see Supplementary Movie S1 at JXB online). Pollen tube growth could be followed until the final stages of growth along the stigmatic papillae and prior to penetrating more deeply into the transmitting tissue (TT). This initial pollen tube growth on the stigmatic papillae takes about 1 h and pollen tubes can grow at rates of 5.86 μm min⁻¹, which is significantly faster than the growth rates observed in optimized in vitro germination mediums. During this phase, pollen tubes could be observed funnelling into the TT (Fig. 2E; see Supplementary Movie S2 online). Pollen tube growth in the stigmatic papillae seems to be relatively homogeneous and synchronous. A fluorescent bulge usually emerges from one of pollen grain apertures close to the contact with the papillae cell and rapidly leads to a short pollen tube that grows relatively fast along or just beneath the papillae cell wall (Fig. 2B–D; see Supplementary Movie S1 at JXB online). Pollen tube growth could be followed until the final stages of growth along the stigmatic papillae and prior to penetrating more deeply into the transmitting tissue (TT). This initial pollen tube growth on the stigmatic papillae takes about 1 h and pollen tubes can grow at rates of 5.86 μm min⁻¹, which is significantly faster than the growth rates observed in optimized in vitro germination mediums. During this phase, pollen tubes could be observed funnelling into the TT (Fig. 2E; see Supplementary Movie S2 online).
Fig. 2. Two-photon imaging of the in vivo pollen tube growth process in the Arabidopsis pistil (see Supplementary Movies S1, S2, and S3 for the entire process). (A) Two-photon live imaging setup. The whole plant was brought to the microscope, the pollinated pistil was mounted in a microchamber and pollen tube growth was imaged in real time. Pollen from a Lat52::eGFP transformed Arabidopsis was used. Pollen grain hydration and germination can be followed over time with high resolution due to the strong GFP signal. After imaging, the pistil was released from the glass slide and the whole plant was brought into the greenhouse to allow recovery. Pistil elongation in the days that followed indicated normal fertilization and seed set, consistent with the non-invasive nature of the imaging process. (B) Overview of a pollinated stigma, where germinated Lat52::eGFP pollen grains (pg) and pollen tubes (pt) can be observed growing along or beneath the papilla (pa) cell surface. The image corresponds to three-dimensional (3D) maximal projection of a Z-series of 2 μm optical sections of a 250 μm stigma sector. The autofluorescence observed on the stigmatic papillae and on the surrounding tissues, allows a good visualization of the structures and the positioning of the pollen grain on the papillae cell. Bar=50 μm. (C) Selected images (at 135 s intervals) from a time-lapse sequence of pollen tubes (pt) germinating on the stigma taken at 45 s intervals over 20 min. Each image represents a 3D maximal projection of 2 μm sections over a 100 μm stigma sector. Arrows indicate emerging or growing pollen tubes.
S2 at *JXB* online), indicating a constraint on the pollen tube growth pathway at this level.

The relatively thick and optically dense tissues surrounding the style transmitting tissue (TT) precludes GFP from the penetrating pollen tubes to be detected and monitoring of the pollen tube growth process usually resumes 3.5–4 h after pollination when the pollen tubes emerge from the internal septum wall of the ovary locules (Fig. 3A). Given that the style length in *Arabidopsis* measures approximately 400 μm and that pollen tubes take around 2.5–3 h to reach the base of the style, the average pollen tube growth rate is estimated at around 2.2–2.7 μm min⁻¹, less than half of the initial growth rate on the stigma papilla. This might not be surprising in solid style plants such as *Arabidopsis*, where pollen tubes have to make their way within intercellular spaces (Lennon *et al.*, 1998), as opposed to growing over the surface of the stigma papillae, or inside the ovary. It should also be noted that the TT is not just passively supporting pollen tube growth but that the secretory cells provide nutrients to facilitate the growth process during the heterotrophic phase (Herrero and Hormaza, 1996). Several studies have demonstrated that molecules present at the TT may be involved in pollen tube adhesion, regulating pollen tube growth or providing ‘competence’ for pollen tubes to respond to guidance cues inside the ovary (Cheung *et al.*, 1995; Higashiyama *et al.*, 1998; Mollet *et al.*, 2000; Park *et al.*, 2000; Kim *et al.*, 2003; Lord, 2003).

Based on the present set-up and optics, the limits of tissue penetration typically reached no more than 120 μm from the surface. This and a number of other biological limitations (see the Supplementary Information for details at *JXB* online) do not permit three-dimensional (3D) resolution of the full ovary. Nevertheless, the fertilization process could be followed in intact ovaries when observations were obtained from pollen tubes that followed a relatively straight growth path close to the carpel surface (Fig. 3A; see Supplementary Movie S3 at *JXB* online). These observations provide useful information about the dynamics of pollen tube growth and guidance as, for instance, can be seen in Supplementary Movie S3 at *JXB* online where a pollen tube tentatively bent three times until finding and gaining access into a virgin ovule, while a second went straight to it. These observations are consistent with the take-over of an ovule by a pollen tube which either blurs the guidance cues or produces a blockage signal to a late arriving pollen tube, prompting it to continue growth until reaching another virgin ovule.

At focal planes where pollen tubes are seen emerging from the internal placental tissue of the septum in conditions detected by vital TPE, autofluorescence from internal organs, including the ovule, funiculus, and the embryo sac is minimal (Fig. 3A). Pollen tubes appear with a bright and intense signal which is detected along the septum, on the ovule funiculus and, in later stages, on the micropyle of the ovules and embryo sacs along the entire ovary (Fig. 3B). Full sequences of optical sections from the epidermis to septum taken from focal planes where the funiculus and ovules become visible are shown in Supplementary Figs S1 and S2 at *JXB* online. In later stages, when a considerable number of pollen tubes are growing inside the ovary, the funiculus and ovules become structurally more visible, and the first fertilized ovules can be detected on the top of the gynoecium due to a well-defined, bright and intense signal present at the micropyle pole of the embryo sac, corresponding to the discharge of the pollen tube contents into the synergid (Fig. 3C). Meanwhile, the embryo sac structure inside the ovule becomes delineated by a more defined autofluorescent signal that is spatially distinct from the eGFP signal discharged by the pollen tube cytoplasm and is also seen in unfertilized ovules.

The instrumental and experimental application of TPE microscopy is still limited by a number of factors, and thus it is probably not realistic to expect that it may become a routine technique in most laboratories to, for example, analyse guidance mutants or be applied as a mutant screening method. However, we believe that, with the ongoing advances in laser and detector sensitivity, together with further enhancement and development of appropriate markers, this methodology may be developed ultimately to allow the observation of cellular events and subcellular dynamics occurring during the pollen tube growth process inside the pistil and to provide relevant structural and physiological data.

### Pollen tube guidance to the female gametophyte

Studies to reveal the guidance mechanisms that direct pollen tubes from the stigmatic surface to the female gametophyte have supported the operation of chemical and physical guidance mechanisms at distinct phases of the *in vivo* pollen tube growth process in different plant species (Hepler *et al.*, 2001; Johnson and Preuss, 2002; Higashiyama *et al.*, 2003;
Several molecules demonstrating tropic activities on the stigma and in the TT have been reported. Lipid molecules, in particular cis-triacylglycerides, have been shown to guide pollen tubes into the stigmatic papillae of tobacco (Wolters-Arts et al., 1998). In the solid style of tobacco flowers, the glycoprotein TTS forms a protein-bound sugar gradient in the TT extracellular matrix, promotes pollen tube growth and attracts them in vitro (Cheung et al., 1995; Wu et al., 1995). Interestingly, pollen tubes secrete glycosylases to remove sugar moieties on TTS, so essentially are capable of sharpening the local TTS protein-bound sugar gradient as they elongate within the style and ovary (Fig. 3).

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pistil. A copper-containing protein chemocyanin has been isolated from the open stylar transmitting canal of lily and attracts pollen tubes grown in vitro (Kim et al., 2003). Moreover, a cysteine-rich lipid transfer protein-like molecule in conjunction with pectin provides adhesion of pollen tubes to the extracellular matrix and may promote directional guidance for these tip growth cells (Mollet et al. 2000).

Directional guidance of pollen tubes is perhaps the least subtle during its approach and entrance to the ovule to gain penetration of the female gametophyte. A series of studies based on Arabidopsis mutants defective in sporophytic and gametophytic tissues in the ovules led to the suggestion that pollen tube guidance to the female gametophyte occurs in two distinct phases: sporophytic and adhesive guidance for growth along the funiculus towards the micropyle, followed by micropyle-guided penetration of the ovule in response to tropic signals that emanate from the female gametophyte (Palanivelu and Pruss, 2006). Genetic studies in Arabidopsis suggest a gradient of γ-amino butyric acid, known to have various signalling and metabolic roles in prokaryotes and eukaryotes, may be involved in pollen tube guidance to the micropyle (Palanivelu et al., 2003). Moreover, nitric oxide (NO) was also shown to have clear negative tropic effects in vitro and a possible role under in vivo conditions, possibly by modulating Ca2+ responses (Feijó et al., 2004; Prado et al., 2004, 2008).

That the synergid cells within the female gametophyte (Fig. 1B) are the primary source for the chemo-attraction of pollen tubes is most dramatically illustrated in Torenia fournieri whose embryo sacs protrude from the ovules and allow laser ablation of cells within the female gametophyte and imaging of the approaching pollen tubes (Higashiyama et al., 2001). Systematic ablation of the seven cells within the embryo sac showed clearly that a single synergid cell is necessary and sufficient for pollen tube attraction to the female gametophyte. Mutations in an Arabidopsis female gametophyte expressed transcription factor, MYB98, also provide a clear delineation between sporophytic guidance along the funiculus and the synergid-based gametophytic guidance for pollen tube penetration of the embryo sac (Kasahara et al., 2005). MYB98 mutants show morphological defects confined to the synergid cell walls. In particular, the filiform apparatus on the micropylar ends of the synergid cells lacks the extensive interlacing formed between synergid cell membrane and cell wall materials characteristic of the wild-type filiform apparatus. Pollen tubes exit the TT and grow normally along the funiculus but fail to enter the micropyle, resulting in severely suppressed seed sets, consistent with defects in guidance mechanisms that are dependent on proper synergid function, possibly in the secretion and production of the filiform apparatus.

Small secreted proteins as guidance molecules from the female gametophyte

Comparative transcript profiling between wild type, myb98 mutant ovules, and ovules from another mutant dif1, which lack the entire embryo sac (Bhatt et al., 1999), identified 400 genes that are down-regulated in the dif1 ovules (Jones-Rhoades et al., 2007). A subset of these is not found among the myb98 transcriptome; among them, several are transcribed in the synergid cells suggesting potential roles for these gene products in synergid cell-mediated pollen tube interaction with the female gametophyte. An astounding 78% of the embryo sac-dependent transcripts are predicted to encode secreted proteins. Interestingly, many of these proteins are related and belong to low molecular weight cysteine-rich defensin-like protein families. Chemotropic molecules are believed to act at short-range to attract cells along a concentration gradient that emanates from the source. Thus use of secreted and low molecule weight molecules as potential attractants is highly plausible and borne true by a more recent study in Torenia. Following from their previous study demonstrating that pollen tube attractants emanate from the synergids cells, Okuda et al. (2009) isolated synergid cell protoplasts from the exposed embryo sacs for cDNA library construction and identified transcripts encoding secreted proteins that may, potentially, have chemotropic activities towards pollen tubes. Amazingly, 29% of the >2000 clones that have been sequenced encode 16 cysteine-rich defensin-like peptides, reflecting a high abundance of these proteins produced by the synergid cells. Two of these, LURE1 and LURE2, when produced from E. coli, efficiently attract Torenia pollen tubes grown under semi-in vivo conditions. Moreover, morpholino antisense LURE oligonucleotides significantly reduce pollen tube entrance to the embryo sac. LURE proteins are highly expressed in the synergid cells, which apparently accumulate many different kinds of cysteine-rich proteins. Okuda et al. (2009) postulated that the high concentrations of LUREs may be important for gradient formation and the variety of cysteine-rich proteins found in the vicinity of the micropyle may have evolved with ovule’s defence system.

In maize, RNAi or antisense knockdown of ZmA1, a 94 amino acids ZmA1 (egg apparatus 1) with a predicted transmembrane domain, resulted in pollen tubes failing to enter the transformed ovules, supporting short-range signaling of pollen tube guidance into the embryo sac (Marton et al., 2005; Dresselhaus, 2006). Detection of cell wall-localized ZmA1-GFP towards the micropylar end of the ovule led the authors to suggest proteolytic removal of the ZmA1 transmembrane domain to permit secretion to the cell wall. ZmA1 is exclusively and highly expressed in the egg apparatus (the egg cell and two synergids) before fertilization, but its transcript level drops precipitously upon fertilization, plausibly to achieve down-regulation of the pollen tube guidance capacity upon fertilization and contributing to the overall strategy to avert polyspermy.

Identification of these female gametophyte-produced guidance molecules are possibly the beacon of knowledge to emerge for pollen tube guidance strategies that have evolved in different plant species. An efficient pollen tube growth and directional guidance assay system (Cheung et al., 1995; Higashiyama et al., 1998; Palanivelu and
Pollen tube penetration of the female gametophyte is followed shortly by tube rupture and discharge of its cytoplasm releasing the sperm cells for fertilization, which in Arabidopsis may be as short as about 1 min (Rotman et al., 2003; Palanivelu and Preuss, 2006; Fig. 3). In most angiosperms, including Arabidopsis, ovules are penetrated by a single pollen tube and late arriving ones either do not approach the penetrated ovules or are actively repelled by them (Fig. 3A; see Supplementary Movie S3 at JXB online; Shimizu and Okada, 2000; Palanivelu and Preuss, 2006). These results are taken as supporting the presence of strategies to avoid polyspermy which, in preventing polyploidy would ensure zygote fitness whereas directing pollen tubes away from fertilized ovules would optimize fertilization of all the available female gametophytes. In the Arabidopsis female gametophyte mutants magatama and 3, micropylar guidance of pollen tubes is defective while the sporophytic guidance mechanism directs pollen tubes to approach the female gametophyte but they cannot target the micropyle, resulting in random growth around and past ovular entrance (Shimizu and Okada, 2000). Moreover, the frequency of a multiple pollen tube approach to a single ovule is significantly increased suggesting that pollen tube penetration triggers a mechanism that would prevent the entrance of late arriving pollen tubes. On the other hand, mutations in the Arabidopsis female gametophyte-expressed, FERONIA/SIRENE receptor kinase, relax this prohibition and result in multiple pollen tube penetration of an ovule but these pollen tubes fail to rupture inside the embryo sac, resulting in severely reduced female fertility (Rotman et al., 2003; Huck et al., 2003; Escobar-Restrepo et al., 2007). Pollen tube guidance mechanisms are apparently intact in these mutants, and the observed phenotypes would suggest that pollen tube rupture is possibly the trigger to deploy strategies that would prevent supernumerary pollen tube penetration and avert polyspermy. Supernumerary pollen tube penetration and their continued growth within the female gametophyte is also induced by mutations in a synergid cell-expressed GPI (glucosylphosphatidylinositol)-anchored cell surface-located protein, LORELEI, further supporting a role for the female gametophyte in regulating sperm discharge (Capron et al., 2008). Interestingly, mutants doubly defective in two, male gametophyte-pre-dominant homologues of FERONIA, ANXUR1 and 2, show premature pollen tube rupture and male infertility (Miyazaki et al., 2009; Boisson-Dernier et al., 2009). It appears therefore that ANXURs and FERONIA may be engaged in an interplay whereby ANXURs ensure pollen tube tip integrity until it encounters the female gameto-

Pollen reception or rejection by the female tissues is critical for reproductive success, species preservation and diversification. Recognition of self and non-self in the pollination and fertilization process reflects evolution of cell-cell communication strategies. Studies that focused on the agronomical importance of pollen-pistil interaction and those that examined the histological and cytological bases of pollination and fertilization have provided much of the framework that underlies our current understanding of the genetic, molecular, and cellular regulation of these processes. For compatible pollination and fertilization, many important molecules, signalling and cellular pathways that regulate the pollen tube growth process have been identified. Identities of regulatory factors from female sporophytic and gametophytic tissues are being elucidated. While additional players apart from those currently known undoubtedly remain to be identified, some of the future efforts will clearly be centred on identifying signal–receptor relationships that mediate male–female interactions during the different phases of the reproductive process. The cellular dynamics that underlie the pollen tube tip growth process is reasonably well-described but its regulation is far from being understood (Cheung and Wu, 2008). Development of molecular, imaging, and computational tools to provide precise temporal and spatial delineation of the relationship between cellular and growth dynamics will be necessary to obtain an accurate understanding of the pollen tube growth process. Information and experimental capabilities derived from these efforts will ultimately enable us to dissect how pollen tubes orchestrate its cellular processes in response to the growth environment in the pistil. The technology described here for imaging pollen tube growth in the pistil, the whole-systems approaches to identify the potential players involved, and the systematic efforts to unravel mechanisms that underlie key events in pollination, pollen tube growth, and fertilization need to be furthered ultimately to enable a precise understanding of the pollen tube growth journey in the pistil and how it may be manipulated for agricultural advantage and ecological balance.

Supplementary data
Supplementary data are available at JXB online.

Supplementary Information. Two-photon imaging of a living pistil and technical considerations.

Supplementary Fig. S1. The montage represents a complete sequence of optical sections (2 μm) of a z-stack from the epidermal surface (ep) to a median section inside the ovary (84 μm).

Supplementary Fig. S2. The montage represents a complete sequence of optical sections (2 μm) of a z-stack from...
the epidermal surface (ep) to a median plane inside the ovary (32 µm).

Supplementary Movie S1. Pollen germination and pollen tube penetration of the stigma.

Supplementary Movie S2. Pollen tube growth in the stylar transmitting tissue.

Supplementary Movie S3. Pollen tube growth in the ovary chamber.

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References


