REVIEW PAPER

Insights into secrets along the pollen tube pathway in need to be discovered

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Abstract

The process of plant fertilization provides an outstanding example of refined control of gene expression. During this elegant process, subtle communication occurs between neighboring cells, based on chemical signals, that induces cellular mechanisms of patterning and growth. Having faced an immediate issue of self-incompatibility responses, the pathway to fertilization starts once the stigmatic cells recognize a compatible pollen grain, and it continues with numerous players interacting to affect pollen tube growth and the puzzling process of navigation along the transmitting tract. The pollen tube goes through a guidance process that begins with a preovular stage (i.e. prior to the influence of the target ovule), with interactions with factors from the transmitting tissue. In the subsequent ovular-guidance stage a specific relationship develops between the pollen tube and its target ovule. This stage is divided into the funicular and micropylar guidance steps, with numerous receptors working in signalling cascades. Finally, just after the pollen tube has passed beyond the synergids, fusion of the gametes occurs and the developing seed—the ultimate aim of the process—will start to mature. In this paper, we review the existing knowledge of the crucial biological processes involved in pollen–pistil interactions that give rise to the new seed.

Keywords: Double fertilization, pollen–pistil interaction, pollen tube guidance, receptors, sexual plant reproduction, signalling molecules.

Introduction

When writing to his friend Joseph Hooker about the puzzling phenomenon of the angiosperm fossil record, Charles Darwin referred to an ‘abominable mystery’: how did the dominance of the angiosperms emerge and how did flowers, their reproductive units, help them to thrive and evolve (Crepet, 2000)? Even now, this long-lasting mystery still remains to be solved. The information that we have already gathered about the angiosperms life cycle has helped us to better understand this

phenomenon, but new players are continually being found that interact during the sexual plant reproduction process to integrate the overall network of the male–female cross-talk that leads to the generation of new seeds.

We can liken our study of angiosperm reproduction to deciphering a map in a treasure hunt, where the promised ‘treasure’ is the seeds that will form the next generation and ensure the continuation of the species. Along the way, we need

to gather clues and trace the pathways in order to untangle the ‘abominable mystery’.

It is always a good idea to begin with the basics: angiosperms change from a sporophytic diploid generation to a gametophytic haploid generation. Inside the flowers, meiotic divisions in stamens and ovules generate two different types of spores, the microspores and the megaspores, respectively. After mitotic divisions, these spores produce the gametophytes that will produce the gametes. The double-fertilization event occurs when the two identical male gametes fuse with the two female gametes, the oosphere and the central cell, thus establishing a new diploid sporophytic generation and completing the plant's life cycle (Yadegari and Drews, 2004).

Arabidopsis thaliana is an important, well-studied model plant, because it is small, with a well-sequenced genome, and a rapid life cycle (Meinke, 1994). Inside the *Arabidopsis* carpel, ovules are enclosed within the ovary, and in each ovule there is a single megasporocyte that undergoes meiosis and produces four haploid megaspores. Only one of these survives to produce the functional megaspore (FM), the others dying as a result of programmed cell death (PCD). After three successive mitotic divisions, the FM forms at the chalaza a female polygonum gametophyte with seven cells and eight nuclei—the embryo sac (Yadegari and Drews, 2004; Palanivelu and Tsukamoto, 2012). At the micropylar pole near the opening of the two integuments, two synergids with their filiform apparatus (resulting from the complex invagination of the synergid cell walls) are present surrounding the egg cell. At the opposite (chalazal) pole there are three antipodal cells, and the central zone is occupied by the central cell with two polar nuclei, which fuse before the arrival of the pollen tube (PT). Over 70% of the flowering plants share this type of female gametophyte (Maheshwari and Johri, 1950; Yadegari and Drews, 2004; Zhou and Dresselhaus, 2019).

The male gametophytes (also known as pollen grains) are formed inside the anther. The mature pollen grain initially has two haploid cells: a vegetative and a generative cell. The generative cell undergoes mitosis, thus originating the two male gametes. In most plants, this mitosis occurs during pollen-tube growth, but in *Arabidopsis* it occurs while the pollen grain is still within the anther. The vegetative cell forms the PT that carries and releases the male gametes inside the female gametophyte (McCormick, 1993, 2004; Lord and Russell, 2002).

Drawing the treasure map—double fertilization

The aimed treasure—the new seed generation—is only formed in flowering plants as a result of the double-fertilization process, the unique key biological feature that has granted the angiosperms their success. The pathway begins once the stigmatic cells recognize a compatible pollen grain, at which point it becomes properly hydrated and forms the PT that will carry the two sperm cells into the ovule embryo sac (Fig. 1). The rapid and controlled navigation of the PT is enabled by a series of communications and consequent signalling cascades that lead to various interactions between the PT and the sporophytic

Fig. 1. Schematic representation of the journey of a pollen tube through an *Arabidopsis* pistil. st, stigma; ov, ovules; pg, pollen grain; pt, pollen tube; tt, transmitting tissue.

and gametophytic female tissues. The PT grows through the extracellular matrix of cells of the female reproductive tissue, namely the stigma, style, and transmitting tissue (TT) (Yadegari and Drews, 2004), until it receives the signals that direct it into an ovule. Once close to the ovule, the PT emerges from the septum and the double-fertilization begins. Having grown along the funiculus, the PT finally reaches the entrance to the embryo sac, the micropyle, and then passes through the filiform apparatus of one of the two synergids, where it stops growing, ruptures, and releases its two sperm cells. It is now time for the process of simultaneous fertilization to occur, where one sperm cell fuses with the egg cell to generate the embryo, while the other fuses with the central cell to form the endosperm, the basal tissue that will provide nourishment to the embryo (Palanivelu and Tsukamoto, 2012; Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Pereira *et al.*, 2014). After PT burst, the penetrated synergid dies by PCD (Higashiyama *et al.*, 2001; Zhou and Dresselhaus, 2019; Dresselhaus and Franklin-Tong, 2013), and the remaining synergid will fuse with the endosperm after successful fertilization of both the female gametes.

This process of organogenesis involves refined control of gene expression, based on subtle communication occurring between neighbouring cells and on chemical signals that induce cellular mechanisms of patterning and growth, all of which are tightly constrained in a complex regulatory network. In this review, we will examine every individual step from the adhesion of the pollen grains to the stigma onwards that leads to the generation of the new seed, mapping the role of each player in each tissue in order to untangle the complex regulatory network of interacting genes.

Step by step: the clues

First interaction of the pollen and stigma

When a pollen grain lands at the stigma cells, it has to immediately face self-incompatibility (SI) responses, a pre-fertilization

barrier aimed at avoiding the negative effects associated with inbreeding depression (Losdat *et al.*, 2014) (Fig. 2). Selective inhibition begins in order to prevent self-fertilization and thus to grant the gene pool and genetic variation of plants (Nasrallah, 2017; Bedinger *et al.*, 2017). This process is controlled by the multiallelic *S*-locus in many plant species (Fujii *et al.*, 2016).

The complex exocytic subunit Exo70A1 in the Arabidopsis stigma accepts a compatible pollen grain and acts to cause polarized secretion from the stigmatic cells and is proposed to provide vesicles containing aquaporins, which improve the water permeability of the pollen grain, allowing it to hydrate, and also provide cell wall-modifying enzymes that allow the PT to grow into the stigma (Safavian *et al.*, 2015; Goring, 2017). Phosphatidylinositol-4-phosphate (PI4P) is crucial in this initial step, as evidenced by the fact that mutants with low levels of PI4P on the stigma have slower hydration rates and maternal fertility defects due to failed pollinations (Chapman and Goring, 2011).

The Arabidopsis VACUOLAR SORTING 41 protein (AtVPS41) is also a controlling factor, as the PTs of *atvps41* plants do not enter the female TT due to a compromised endocytic pathway (Hao *et al.*, 2016). Ca^{2+} is an important intracellular signalling factor for the formation of the PT and its polarized growth (Iwano *et al.*, 2004; Pereira *et al.*, 2016a).

The mysterious arabinogalactan protein (AGP) family, which is always involved in distinct stages of the reproductive process, may also be essential regulatory components of PT growth (Lamport *et al.*, 2018). The receptivity of apple blossom stigma depends on secretion of AGPs and their disappearance is linked with PT growth (Losada and Herrero, 2012). The molecular properties and periplasmic location of the classical AGPs (Lamport *et al.*, 2006) make them the primary source of oscillations in cytosolic Ca^{2+} , pectic plasticizers, working as Ca^{2+} signposts to the ovule (Lamport *et al.*, 2018). AGPs present in the stigma cells and in growing PTs have roles in pollen–stigma interactions and pollen grain competence that

initiate PT growth. The pollen-specific AGP6, AGP11, AGP23, and AGP40, are included in the PT endosome machinery (Costa *et al.*, 2013; Pereira *et al.*, 2016a).

The gene *O-FUCOSYLTRANSFERASE1* (*AtOFT1*) plays a key role in PT penetration through the stigma–style interface and, based on genetic and physiological evidence, a Golgi-localised fucosyltransferase system may be needed for PT growth through the pistil tissues. Although as yet unknown, the substrates of *AtOFT1* may include cell-surface receptors or structural proteins secreted into the extracellular matrix. A nearly sterile *oft1* phenotype should provide a means to determine how putative proteins OFTs are utilized within pollen–pistil interactions (Smith *et al.*, 2018). Fasciclin-like AGPs (FLAs) are also recruited during this process, as FLA3 is expressed in the pollen grains and PTs and is involved in the development of microspores by affecting cellulose deposition (Li *et al.*, 2010).

The puzzling navigation of the pollen tube along the transmitting tissue

The rapid and controlled growth of the PT is enabled by an accurate communication network and signalling cascades during the pollen–pistil interactions (Figs 3, 4). The PT guidance process starts with ‘preovular guidance’, where any compatible PT travels in the extracellular matrix of the TT or channel from the stigma to the ovary without any influence from the target ovule, and later turns to ‘ovular guidance’ in which a specific relationship is formed between one PT and its ovule (Higashiyama and Takeuchi, 2015; Kanaoka and Higashiyama, 2015; Mizuta and Higashiyama, 2018).

Mechanical, chemotropic, geometric, and physiological orientation mechanisms may explain the precise growth of PTs towards the ovary (Heslop-Harrison, 1987; Lush, 1999; Cameron and Geitmann, 2018). There is a strict balance between cell turgor and cell wall resistance to maintain the shape

Fig. 2. The players involved in pollen–stigma interactions and their roles. PT, pollen tube; TT, transmitting tissue.

Fig. 3. The players involved in the preovular guidance stage and their their roles. PT, pollen tube.

Fig. 4. The players involved in navigation of the pollen tube (PT) along the transmitting tissue (TT) and their roles.

of the PT, enabling the tip to expand and grow and preventing the loss of the cell integrity (Winship *et al.*, 2011). Accurate cell wall assembly is fundamental for PT growth. The polysaccharide pectin, an essential component of the primary cell wall, interferes with the wall structure and flexibility during cell growth. Pectin methylesterase (PME) enzymes spatially and temporally control the de-esterification of pectin, essential for PT integrity. PTs become unstable when losing de-esterified pectin. Arabidopsis *Vanguard1* (*VGD1*) encodes a pectin methylesterase, and the *vgd1* mutant helps to demonstrate the importance of pectin in the cell wall for PT integrity as it presents cell bursting *in vitro* and delayed growth *in vivo* (Bosch and Hepler, 2005; Jiang *et al.*, 2005). In addition, the Cl⁻ channel MSL8 in Arabidopsis has been shown to cause precocious bursting of the PT before arriving at the synergids (Li and Yang, 2018). Enzymatic production of reactive oxygen species (ROS) also affects PT growth. Characterization of the NADPH oxidases RbohH and RbohJ (Respiratory burst

oxidase homologs H and J) in Arabidopsis has shown that the tip growth of the PT is severely impaired in the *rbohH rbohJ* double-mutant. Both RbohH and RbohJ, which have Ca²⁺-binding EF-hand motifs, show Ca²⁺-induced ROS-producing activity at the PT tip of the plasma membrane. The extension of the PT slows down to couple the rates of exocytosis and cell expansion, possibly through Ca²⁺ influx and K⁺ efflux (Kaya *et al.*, 2014; Lassig *et al.*, 2014). The affected amplitude and frequency of ion oscillation are detrimental to the precise coupling of the tip growth and the cell wall dynamics. The integrity of the PT depends on the cell wall composition and is controlled by its modifying enzymes and ROS, except for extensins (Jiang *et al.*, 2005; Woriedh *et al.*, 2013).

In *Torenia fournieri*, the PT has a unidirectional orientation (Higashiyama and Hamamura, 2008), and possibly does not need the female gametophyte (FG) for the growth of the PT from the stigma to the style (Sogo and Tobe, 2006). With preovular guidance in mind, chemocyanin in the stigma is found

to induce PT chemotropism in *Lilium longiflorum*, with stigma cysteine-rich adhesins (SCAs) binding with the PT and allowing the chemocyanin to have physical access to the plasma membrane, thus controlling and directing the growth of the PT (Park *et al.*, 2000; Kim *et al.*, 2003). PT pectins may interact with the Arabidopsis LIPID TRANSFER protein 5 (LTP5) to facilitate guidance (Chae *et al.*, 2009). In tomato, CYSTEINE-RICH STIGMA SPECIFIC PROTEIN 1 (STIG1) interacts with a KINASE 2-type pollen receptor (LePRK2) and with phosphatidylinositol 3-phosphate (PI3P) as a signal peptide to promote PT growth (Tang *et al.*, 2004); In Arabidopsis, PT growth is promoted by pistil brassinosteroids (Vogler *et al.*, 2014), with γ -aminobutyric acid (GABA) modulating the Ca^{2+} channels in the plasma membranes, thus regulating the PT signalling pathways (Palanivelu *et al.*, 2003; Yu *et al.*, 2014). During preovular guidance (Fig. 3), the growth of the PT is therefore enabled by several mechanisms (Mizuta and Higashiyama, 2018).

The TT that connects the stigma, style, and ovules supports the growth of PTs through an extracellular matrix (ECM) that is extremely rich in glycoproteins, polysaccharides, glycolipids, and AGPs (Pereira *et al.*, 2015). The Arabidopsis TT is solid and composed of cylindrical cells (Lennon *et al.*, 1998; Erbar, 2003) that facilitate PT development and nutrition (Faure *et al.*, 2002; Crawford and Yanofsky, 2008). According to hormonal studies, the establishment of the TT may be controlled by both auxin and cytokinin, which are responsible for the tissue polarity (Deb *et al.*, 2018) and thus may play a key role in the complex interactions that control PT growth.

In *Nicotiana tabacum*, the highly glycosylated TRANSMITTING TISSUE SPECIFIC (TTS) AGP proteins promote PT growth and guidance to the ovules (Cheung *et al.*, 1995; Wu *et al.*, 2001). Transcription factors (TFs) such as NO TRANSMITTING TRACT (NTT), HECATE 1 (HEC1), HEC2, HEC3, HALF FILLED (HAF), SPATULA (SPT), and water-soluble chlorophyll proteins (AtWSCP) are important for TT development because they induce the PCD of the tissue after the PT has passed, which is also crucial for PT growth (Alvarez and Smyth, 2002; Crawford and Yanofsky, 2011; Boex-Fontvieille *et al.*, 2015; Mizuta and Higashiyama, 2018). *ntt* mutants show abnormal development of the TT, together with defects and slower growth of the PT, and a reduced fertilization rate (Crawford *et al.*, 2007). In addition, *ntt* mutants have lower levels of acidic polysaccharides, which is possibly related to AGP content as they are acidic glycoproteins associated with the TT.

The PT may be guided by distinct cues, including K^+ , Cl^- , Ca^{2+} (Hepler *et al.*, 2006), glycoproteins (Sommer-Knudsen *et al.*, 1998), ROS (Foreman *et al.*, 2003), nitric oxide (Prado *et al.*, 2008), and peptides (Qu *et al.*, 2015) as well as other complex signalling networks that have not yet been elucidated (Leydon *et al.*, 2015).

In Arabidopsis, the Shaker family K^+ channel, SPIK, affects PT development. Loss of SPIK leads to precocious bursting of the PT together with a delayed growth, suggesting a conserved role of K^+ homeostasis in PT growth and bursting (Mouline *et al.*, 2002).

The presence of AGPs along the pathway from the stigma to the egg cell indicates that they also provide guidance cues

(Pereira *et al.*, 2014, 2016a). If pollen tubes grown *in vitro* acidify their growth medium (Feijo *et al.*, 1995), it may indicate that *in vivo* they can dissociate AGP-Ca^{2+} of the TT, enabling the PT to create a Ca^{2+} path to the ovule. The fertilization of multiple ovules can also be enabled by the cooperation of multiple PTs (Heslop-Harrison *et al.*, 1985) together with an increase of H^+ efflux and the release of Ca^{2+} from locally abundant AGP-Ca^{2+} (Lampert *et al.*, 2018).

High levels of expression of AGP1, AGP4 (JAGGER), and AGP19 can be found along the TT in Arabidopsis (Yang *et al.*, 2007; Pereira *et al.*, 2014, 2016a, 2016b). AGP19 has high expression in the style, ovary walls, TT, and siliques, and *agp19* mutants have low flower production and smaller siliques (Yang *et al.*, 2007). AGP12 and AGP15, which are also located along the PT pathway, can contribute nutritionally to its growth from the top of the stigma to the base of the pistil, and facilitate its movement, provide guidance to its targets, and possibly making it competent for being received by the embryo sac (Pereira *et al.*, 2014).

An AGP side-chain fragment designated AMOR is the PT activation molecule for the response to the LURE guidance peptides of the synergids in *Torenia* (Okuda *et al.*, 2009). AMOR has been identified as a methyl-O-glucuronosyl-b-D-galactose (Mizukami *et al.*, 2016), possibly a key component of the AGP Ca^{2+} -binding motif. AMOR has a differential localization in specific structures in the AG sugar chains, indicating that AG biosynthesis enzymes re control AGP functions and their sugar chains (Su and Higashiyama, 2018).

It is still not entirely clear how all the TT components interact with the PT and, irrespective of the importance of these interactions, the different components present in the TT point to a contact-mediated ability provided by the stigma and style for the PT to become aware of the signals that direct it to the ovule (Palanivelu and Preuss, 2006). The TT is thus a key tissue for the quick growth of the PT through the pistil, but it also provides it with the ability to move accurately towards the ovule (Fig. 4).

Ovular guidance: funicular and micropylar

The PT exits from the TT through a very narrow space between the cells of the septum (Palanivelu and Tsukamoto, 2012), interacting with all the quests and tissues that trigger its emergence (Higashiyama and Takeuchi, 2015), during a crucial transition phase from preovular to ovular guidance (Mizuta and Higashiyama, 2018). When the PT emerges from the septum, it enters a funicular guidance phase from the placenta along the funiculus, and then it enters a micropylar guidance phase where it navigates from the funiculus to the micropyle (Shimizu and Okada, 2000; Mizuta and Higashiyama, 2018). This pathway is dependent on signals from sporophytic tissues and from the female gametophyte (Pereira *et al.*, 2016a) (Fig. 5).

In Arabidopsis, two K^+ transporters present in the PT, CATION HYDROGEN EXCHANGER 21 (CHX21) and CHX23, are able to block growth along the funiculus. *chx21 chx23* double mutant show normal PT growth through the TT, but the tubes are unable to grow into the ovule because they

Fig. 5. The players involved in the ovular guidance stage, divided in funicular and micropylar guidance steps, and their roles. PT, pollen tube; TT, transmitting tissue.

are incapable to perceive the signals that come from the ovules (Lu et al., 2011). PT competence for signalling is also dependent on MITOGEN-ACTIVATED PROTEIN KINASE (MPK3) and MPK6 (Guan et al., 2014), and the *mpk3 mpk6* double-mutant has PTs that are defective in the funicular guidance phase. PTs can only pass through the TT with the controlling help of PHYTOSULFOKINE (PSK), as mutants show very low fertilization rates and loss of funicular orientation (Stührwohldt et al., 2015). Our knowledge of the mechanisms that regulate this stage is increasing but it remains incomplete, and studies are ongoing to determine whether there are additional guidance cues.

ABNORMAL POLLEN TUBE GUIDANCE 1 (APTG1), a mannosyltransferase (Dai et al., 2014), acts in the glycosylphosphatidylinositol (GPI) synthesis of the COBRA-LIKE 10 protein (COBL10) (Li et al., 2013). This protein, located at the tip of the PT, plays a crucial role in its orientation and growth. Mutations in COBL10 generate gametophytic male sterility due to low PT growth and defective directional sensing in the female TT. The formation of the apical pectin cap and the cellulose microfibrils are defective in the PTs of the *cobl10* mutant (Li et al., 2013).

After the funicular guidance period, the PT enters the micropylar guidance stage. On reaching the micropylar region, the PT passes through an opening between the ovule integuments until it reaches one of the synergid cells, which are active and typically secretory cells (Huang and Russell, 1992). The *wuschel-7* (*wus-7*) mutant has a short inner integument (Lora et al., 2019), with 65% of *wus-7* ovules showing no PTs

emergence in the micropyle, and 35% of its ovules show defective embryo sacs. These results suggest that the short inner integument has a partial effect on PT guidance. It has long been known that the presence of a functional FG influences the attraction of the PT to the ovule (Ray et al., 1997; Shimizu and Okada, 2000), and it has now been established that it is the structure that produces the ovular-attraction molecules (Takeuchi and Higashiyama, 2016). Based on evidence from *T. foeniculifer*, the synergids are the female cells responsible for the ovular attraction of the PT. *Torenia* has a bare embryo sac, which is projected to the outside of the ovule through the micropyle when mature, thus allowing easy access to the embryo sac cells. It has been shown *in vitro* that PTs are attracted directly to the bare embryo sacs and hence do not leave the micropylar region, due to the production of attraction signals by the embryo sac (Higashiyama et al., 2001). Laser ablation performed on different gametophytic cells has determined that the synergids provide guidance to the PT via diffusible chemical signals (Higashiyama et al., 1998, 2001; Higashiyama and Hamamura, 2008; Horade et al., 2013).

An equivalent scenario happens in *Arabidopsis* with the MYB98 TF expressed in the synergids. The *myb98* mutant has an abnormal filiform apparatus, which blocks the secretion of attractant molecules and hence the PT cannot find the micropyle (Kasahara et al., 2005; Punwani et al., 2008). The interaction between the egg and central cells is also important for ovular guidance, since laser disruption of the egg cell or the polar nucleus of the central cell can severely inhibit attraction of the PT (Susaki et al., 2015). Thus the synergids act together

with the egg and central cells to regulate the PT attraction during the last stage of its journey (Zhou and Dresselhaus, 2019; Pereira *et al.*, 2016b; Mizuta and Higashiyama, 2018).

Hence there are attractant molecules produced by the synergids, as identified by several studies in different plant species. In *T. foeneri*, the cysteine-rich peptides (CRPs) TFLURE1 and TFLURE2, from the defensin-like protein subgroup, have been identified as attractant molecules that induce micropylar guidance (Okuda *et al.*, 2009), and are capable of also attracting PTs *in vitro* (Higashiyama, 2010). LURES have also been identified in Arabidopsis (AtLURES), *A. lyrata* (AILURES) (Takeuchi and Higashiyama, 2012), and *T. concolor* (TcCRP1) (Kanaoka *et al.*, 2011). In Arabidopsis, these small proteins, which are only expressed in the synergids, are secreted through the filiform apparatus (Takeuchi and Higashiyama, 2012). The mutants *magatama3* (Shimizu and Okada, 2000), *myb98* (Kasahara *et al.*, 2005), and *central cell guidance* (Chen *et al.*, 2007) have been found to be defective in micropylar guidance due to an absence of AtLURE1 expression, indicating that it controls PT attraction (Takeuchi and Higashiyama, 2012). The novel interaction partner of the transcription factor CCG, the CCG-binding protein CBP1, seems to co-regulate the expression level of a subset of CRPs, including LURES, by recruiting the Mediator complex and RNA Pol II with CCG in the central cell (Li *et al.*, 2015). Diverse genes expressed in the central cell and synergid are down-regulated in *ccg* ovules such as *MYB98*, which is responsible for regulation of transcription of *LURES*. Mutation of *MAA3* affects PT attraction in Arabidopsis. *MAA3* encodes a Sen1p-like RNA helicase and is believed to control target mRNA molecules whose products are needed to guide PTs (Shimizu *et al.*, 2008). Moreover, recombinant AtLURE1 peptides expressed in *Torenia* are capable of attracting Arabidopsis PTs when *Torenia* ovules are placed *in vitro* close to the tip of Arabidopsis PTs (Takeuchi and Higashiyama, 2012). These peptides are therefore sufficient to overcome reproductive barriers in ovule orientation even between distant species (Mizuta and Higashiyama, 2018).

Unfertilized Arabidopsis ovules send diffusible attractant molecules (Palanivelu and Preuss, 2006; Takeuchi and Higashiyama, 2012), but these are no longer produced when the ovule is penetrated by a PT, which may in itself be sufficient to prevent the attraction of more PTs. But PTs may also be able to rapidly release diffusible repellents, although there is no firm evidence for this yet (Palanivelu and Preuss, 2006).

The specific *Zea mays* *EGG APPARATUS1* gene (*ZmEA1*) encodes a small protein that is present not only in the synergids but also in the egg cell, the filiform apparatus, and the micropylar nucellus, and is necessary for micropylar orientation (Márton *et al.*, 2005, 2012). No homologous candidate has been found in Arabidopsis, but there is no doubt about the importance of embryo sac cells for successful PT guidance.

GAMETE-EXPRESSED3 (*GEX3*) encodes a membrane protein and is expressed both in male and female tissues. Micropylar PT guidance defects are caused by aberrant expression of *GEX3* in the egg cell (Kanaoka and Higashiyama, 2015).

As evidenced by immunolocalization studies in ovules and the filiform apparatus of synergids of different species, AGPs are also present in these tissues as attractant and/or signalling

molecules for PT guidance (Coimbra and Salema, 1997; Coimbra and Duarte, 2003; Coimbra *et al.*, 2007; Lopes *et al.*, 2016). AGP23 is present at high levels inside the embryo sac upon fertilization where the PT bursts (Pereira *et al.*, 2016a).

The JAGGER protein, AGP4, is essential in preventing the attraction of multiple pollen tubes to a single ovule (known as polytubey). The *jagger* persistent synergid does not undergo PCD and more than one PT is attracted to the ovule, in a polytubey effect. JAGGER controls the persistent synergid death and ends the multiple PTs attraction (Pereira *et al.*, 2016b).

Arabidopsis has LURE1 receptors such as leucine-rich repeat (LRR) kinase-type receptors (RLKs), including MALE DISCOVERER 1 (MDIS1), MDIS1-INTERACTING LIKE KINASE RECEPTOR (MIK1, MIK2) (Wang *et al.*, 2016), and POLLEN RECEPTOR-LIKE KINASE 6 (PRK6) (Takeuchi and Higashiyama, 2016). The recombinant AtLURE1 may induce heterodimerization of MDIS, MIK1, and MIK2 (Wang *et al.*, 2016). These three receptors are located at the PT plasma membrane and knock-out lines exhibit defects in micropylar guidance. The tip-localized pollen-specific receptor-like kinase 6 (PRK6) is essential for the detection of AtLURE1, and hence controls the attraction response and elongation of the PT (Takeuchi and Higashiyama, 2016). The detection of AtLURE1 by PRK6 is achieved in cooperation with other PRK family receptors such as PRK1, PRK3, and PRK8, but PRK6 is the key receptor. PRK6 also interacts with pollen-expressed ROPGEFs (Rho of plant guanine nucleotide-exchange factors), which are important for PT growth through activation of the signalling switch Rho GTPase ROP1 (Kaothien *et al.*, 2005; Zhang and McCormick, 2007; Takeuchi and Higashiyama, 2016).

PT guidance is also affected by nitric oxide (NO) and D-serine. NO has diverse effects on plant development as a growth regulator, and acts as a negative chemotropic cue during *in vitro* PT growth and re-orientation, which is mediated by a cGMP signalling pathway (Prado *et al.*, 2004). Mutations in Arabidopsis *NITRIC OXIDE SYNTHASE1* (*AtNOS1*) affect NO production and result in low fertility (Guo *et al.*, 2003), indicating that NO has potential functions during PT guidance. Experiments with semi-*in vivo* isolated ovules and PTs have confirmed that NO is necessary for ovular PT guidance in *Lilium longiflorum*, with the pathway being dependent on Ca²⁺ signalling (Prado *et al.*, 2008). It remains unclear how the PT surface perceives NO.

The Ca²⁺ signalling network is complex due to the existence of multiple Ca²⁺ channels, distinct regulation levels, and numerous PT Ca²⁺ sensors. Among other Ca²⁺ channels expressed in pollen, the Cyclic nucleotide-gated ion channel 18 (CNGC18) appears to be essential in PT growth and guidance (Frietsch *et al.*, 2007). Examination of two point-mutations of *cngc18* showed that the cyclic nucleotide (cNMP) activation of CNGC18 is probably involved in ovular guidance, but not in PT growth (Gao *et al.*, 2016). Thus, Ca²⁺ influx may regulate the growth and guidance of the PT in a dual manner: basal influx for PT growth, and cNMP-activated influx for PT re-orientation (Li *et al.*, 2018).

LOST IN POLLEN TUBE GUIDANCE 1 (LIP1) and LIP2 are also involved in the AtLURE1-dependent orientation

mechanism (Liu *et al.*, 2013; Pereira *et al.*, 2016a; Higashiyama, 2018). Due to the lack of a specific extracellular region, LIPs do not bind directly to the attractant molecules, including the AtLURE1 peptides, but they may form complexes with direct receptors of the LURE peptides, and thus act in signal transduction in the PT cytoplasm (Takeuchi and Higashiyama, 2016).

It is also important to map distinct proteins from the endoplasmic reticulum (ER), since their folding may be important for PT competence, enabling it to respond to attraction or orientation signals (Braakman and Herbert, 2013). POLLEN DEFECTIVE IN GUIDANCE 1 (POD1) is an ER protein that interacts with the chaperone CALRETICULIN 3 (CRT3) to control folding of the PT membrane proteins and to regulate their signalling (Li *et al.*, 2011). Several molecules act in an intrinsic signalling pathway during the micropylar guidance stage, but knowledge of their composition and interactions is sparse (Pereira *et al.*, 2016a; Mizuta and Higashiyama, 2018) and further research is needed.

The treasure beyond the synergids

At the end of its journey, the PT reaches the micropyle of its target ovule, enters the embryo sac through one of the

synergids, stops its growth, and bursts to release the two male gametes (Leshem *et al.*, 2013) that will fuse with the female ones; meanwhile the systems act to prevent multiple PTs converging on the ovule (Fig. 6). The receptive synergid undergoes PCD shortly before or upon entry of the PT (Faure *et al.*, 2002). Through a complex signalling system and communication between the PT and the female tissues, the ultimate goal of generating a new seed is achieved, and the reproductive success of the plant is enabled (Pereira *et al.*, 2016a).

Synergids gather the utmost point of PT attractant molecules and are able to receive and rupture it. The first PT receptor discovered, from the synergid filiform apparatus, was a serine/threonine-type FERONIA/SIRENE (FER/SRN). The *fer/srn* mutation leads to continuous growth of the PT within the embryo sac, with no release of the male gametes (Huck *et al.*, 2003; Escobar-Restrepo *et al.*, 2007; Rotman *et al.*, 2008).

FER is a receptor-like kinase (RLK) from the *Catharanthus roseus* CrRLK1L-1 subfamily with an extracellular segment similar to a lectin domain, meaning that its binding molecule can be a carbohydrate or a cell wall glycoprotein (Lindner *et al.*, 2012; Pereira *et al.*, 2016a). FER is an essential factor in stopping the growth of the PT, its reception by the synergid, and its correct rupture and release of the gametes, and it may

Fig. 6. The players involved during the interaction between the (PT) and the synergids and their roles.

also be the link between extracellular events and intracellular orientation (Stegmann *et al.*, 2017).

LORELEI (LRE) is a GPI anchor protein predominantly expressed in the synergids and the *lre* mutant has a similar phenotype to the *fer/srn* mutation (Capron *et al.*, 2008). LRE and FER work together in the reception of the PT by the synergid, with LRE acting as a co-receptor with FER (Hafidh *et al.*, 2016; Liu *et al.*, 2016). LRE and LLG1 (LORELEI-like GPI anchored protein 1) interact with FER in the ER lumen and act as chaperones to conduct FER to the filiform apparatus and to regulate rupture of the PT (Li *et al.*, 2015).

NORTIA (NTA) also has a *fer*-like phenotype (Kessler *et al.*, 2010). NTA acts in a FER-dependent way in the same signalling cascade. Its C-terminal calmodulin domain suggests it is able to perceive Ca^{2+} oscillations in the synergids (Iwano *et al.*, 2012). Both the *lre* and *nta* mutants have a similar phenotype to the *fer* mutant, but not fully penetrant as this last one. They play important roles in the PT-synergid interaction, but they may be acting redundantly with another as yet unknown factor that still needs to be identified before we can fully understand this signalling mechanism (Pereira *et al.*, 2016a).

In Arabidopsis, in parallel to the FER signaling pathway, vacuolar acidification in synergid cells is impaired under low expression levels of AP1G, the γ -subunit of the tetrameric ADAPTOR PROTEIN1 (AP1). As a result, there is a delay in synergid cell degeneration upon arrival of the PT. AP1 regulates protein sorting at the trans-Golgi network/early endosome. In synergids cells with functional loss of V-ATPases, the *ap1g* mutant has a defect in the reception of the PT, indicating that acidification of the vacuole represents an important mechanism in synergid degeneration (Wang *et al.*, 2017).

Other female gametophyte CrRLK1L receptors involved in the perception of the PT have been found (Galindo-Trigo *et al.*, 2018, Preprint). HERCULES RECEPTOR KINASE1 (HERK1) and ANJEA (ANJ) are strongly localised at the filiform apparatus of the synergid cells, and the *herk1 anj* double-mutant shows over-growth of the PT inside the female gametophyte without rupture. Yeast two-hybrid assays show that the extracellular membrane domains of HERK1 and ANJ interact with LRE, leading the assumption that HERK1-LRE and ANJ-LRE may belong to a receptor complex in the filiform apparatus that is responsible for mediating the reception of the PT.

TURAN (TUN), encoding a UDP-glycosyltransferase protein, may also be involved in reception of the PT by the synergid, with a mutation that again shows a phenotype with over-growth of the PT inside the female gametophyte without rupture (Lindner *et al.*, 2015). The VERDANDI (VDD) TF belongs to the Reproductive Meristem (REM) family, and *vdd* shows defects in the cellular identity of the antipodals and synergids, with no PT rupture and thus continuation of its growth after reaching the synergids (Matias-Hernandez *et al.*, 2010). Recently, VDD and VALKYRIE (VAL, also a REM family member) have been shown to act as a complex that regulates the death of the receptive synergid, and also the death of the PT when it bursts (Mendes *et al.*, 2016).

A small group of chimeric AGPs, the EARLY NODULIN-LIKE (ENODLs), EN11–EN15, are essential for the reception of the PT in Arabidopsis, with high expression levels found in the funiculus and in the ovules. EN14 and EN15 are present in the synergid filiform apparatus. The quintuple-mutant shows a similar phenotype to the *fer* mutant, with EN15 at least interacting with FER and hence perhaps being involved in the activation of the signalling pathway that is essential for the co-ordination of the male–female communication needed in the double-fertilization process (Hou *et al.*, 2016).

There are also male players involved in the reception by the synergids and the rupture of the PT, clearly interacting in a co-ordinated way with the female players. The first identified set of pollen-specific MYB transcription factors, namely MYB97, MYB101, and MYB120, act to control the reception of the PT and to regulate its gene expression, including genes encoding the secretion of small peptides (Liang *et al.*, 2013).

The Ca^{2+} pump ACA9 expressed in the PT has also been shown to regulate PT growth and rupture in the synergid (Schiott *et al.*, 2004).

ANXUR 1 (ANX1) and ANX2 are receptor-like kinases and FER homologues that induce the PT to burst (Boisson-Dernier *et al.*, 2009). When the receptive synergid goes into PCD, Ca^{2+} responses are activated (Boisson-Dernier *et al.*, 2013; Ngo *et al.*, 2014). The over-expression of ANX1/ANX2 receptors at the plasma membrane of the PT tip leads to its growth being blocked as a result of excessive activation of exocytosis and accumulation of secreted material by the cell wall. The *anx1 anx2* double mutant stops PT development by premature rupturing of the tip (Boisson-Dernier *et al.*, 2013). It has been established that ANX blocks the rupturing of the PT, and hence premature release of the gametes, by maintaining cell wall integrity, so it continues to grow in the female tissues until it reaches the FG (Boisson-Dernier *et al.* 2009; Miyazaki *et al.*, 2009; Pereira *et al.*, 2016a). Muro *et al.* (2018) have shown that the localization of ANX receptors in the PT tip is strictly mediated by two ANTH domain-containing proteins via the clathrin-mediated endocytosis pathway. The *phosphatidylinositol binding clathrin assembly protein 5a* (*picalm5a*) *picalm5b* double-mutant exhibits an altered distribution of ANX receptors in the PT and has an identical phenotype to *anx1 anx2*, i.e. premature bursting of PTs during their growth.

There has to be an extremely coordinated mechanism of signalling between the female and male cells during these interactions. Once in the FG, the FER-dependent signalling cascade located in the synergid is activated to mediate the reception of the PT and the fertilization process, while the ANX-dependent signalling present in the PT is blocked, enabling the PT to rupture and release the gametes (Boisson-Dernier *et al.*, 2009; Miyazaki *et al.*, 2009). This is an interesting example of duplicate sibling genes of an ancestral gene working on cells of the opposite sex during male–female interactions (Higashiyama, 2018).

RAPID ALKALINIZATION FACTORS (RALFs) are secreted peptides that function as extracellular signals and are translated into the cell. These peptides are spread across the plant kingdom and Arabidopsis has 37 members (Murphy

and De Smet, 2014) regulating various physiological processes (Stegmann *et al.*, 2017).

The expression of RALF peptides in PTs was examined by Mecchia *et al.* (2017), who selected RALF4 and RALF19, the closest homologues, as the focus of their study. The *ralf4 ralf19* double-mutant shows a similar phenotype to *anx1 anx2*, i.e. no maintenance of PT growth. Thus, RALF4/19 are crucial for growth of the PT, and possibly act upstream of ANX1/2.

Searching for additional CrRLK1Ls involved in PT reception, Ge *et al.* (2017) found two new receptors predominantly expressed in the PT, namely BUPS1 and BUPS2, and determined that the *bups1 bups2* double-mutant has a similar phenotype to *anx1 anx2*. It is possible to find RALF4/19 interacting with BUPS1/2 and these two peptides interacting with ANX1/2. In addition, BUPS1/2 and ANX1/2 interact with each other. Given that the same premature-rupture phenotype of the PT is shown by the *anx1 anx2* and *bups1 bups2* double-mutants, a model may be suggested in which ANX1/2 and BUPS/2 form a heterodimer. Further studies are needed to determine whether RALF4/19 binding induces heterodimerization of ANX and BUPS. RALF34, expressed only in the female tissues, is the closest homolog of RALF4/19 and induces PT rupture at 2 nM concentration, whereas RALF4/19 show no activity even at 20 µM. RALF34 competes with RALF4/19 *in vitro* in the BUPS1–ANX interaction, and so it can control PT rupture through the female tissues; however, we do not yet understand how this happens, and further studies are needed (Liang and Zhou, 2018).

The autocrine signalling mediated by RALF4/19 and ANX–BUPS is critical for maintaining long-distance growth of the PT and for the cell wall integrity of its tip. Questions remaining to be answered are how the external autocrine and paracrine signals for the PT tip change during its long journey and how the PTs respond to them (Higashiyama, 2018).

The improved understanding of the PT guidance and fertilization process includes a limited number of molecular players. Considering the FER and ANXUR1/2 description and their role in Ca²⁺-mediated fertilization, members of the CrRLK1L family had stepped into the regulation of polarized growth. ERULUS (ERU), also a CrRLK1L protein, has been described

as a core regulator of root hairs and is involved in the establishment of a functional apical [Ca²⁺]_{cyt} gradient (Bai *et al.*, 2014). Pollen of the *eru* mutant are less competitive *in vivo* than wild-type pollen in terms of plant fertilization efficiency (Schoenaers *et al.*, 2017) and show aberrant ovule-targeting of PTs, with more mutant PTs growing around the funiculus with multiple turns before targeting the micropyle. This is a similar phenotype to the *lip1 lip2* double-mutant, which is defective in two PT plasma membrane-localized RLKs (Liu *et al.*, 2013). LIP1 and LIP2 are crucial players in the receptor complex that regulates PT guidance in response to the micropyle-secreted AtLURE1 signalling peptide. It could therefore be suggested that ERU could function as a LURE receptor, and as such it could regulate PT targeting in a complex with LIP1 and LIP2 (Schoenaers *et al.*, 2017).

After all the PT–synergid interactions, the PT ruptures and releases its contents, allowing the fusion of the male and female gametes (Fig. 7). For successful fusion to occur, three crucial players interact: GAMETE EXPRESSED 2 (GEX2), GENERATIVE CELL SPECIFIC 1/HAPLESS 2 (GSC1/HAP2), and EGG CELL1 (EC1) (Mori *et al.*, 2006; von Besser *et al.*, 2006; Sprunck *et al.*, 2012). GEX2 encodes a membrane-expressed protein of the male gametes that contains extracellular immunoglobulin-like domains required for gamete fusion (Mori *et al.*, 2014). When male gametes with the *gcs1 hap2* double-mutation are delivered to the ovules they cannot initiate fertilization, and the released gametes remain in the fusion site together with the female gametes, eventually leading to the attraction of multiple PTs (Mori *et al.*, 2006; von Besser *et al.*, 2006). EC1 is a cysteine-rich protein and is a possible activating molecule of the male gametes that permits fusion. *ec1* quintuple-mutants show that successful male–female gamete interactions prevent multiple sperm-cell delivery (Sprunck *et al.*, 2012).

Respiratory burst oxidase homologue H (RBOHH) and RBOHJ have been found to act downstream of ANXs to control ROS production during PT growth (Boisson-Dernier *et al.*, 2013). The tube-burst phenotype of both the *anx1 anx2* and *rbohH rbohJ* double-mutants can be suppressed by a point-mutation (MRIR^{240C}) in the receptor-like cytoplasmic kinase MARIS (MRI) (Boisson-Dernier *et al.* 2015), while

Fig. 7. The players involved during fusion of the gametes and their roles.

RALF4 and 19 have been shown to function upstream of MRI (Mecchia *et al.*, 2017). Thus, the RALF-BUPS/ANX complex may be regulating downstream RBOHH, RBOHJ, and MRI to maintain the integrity of the PT during its growth. Moreover, RALF4 and 19 bind the cell wall extensins LRX8, LRX9, and LRX11, which are expressed in the PT and have a vital role in the deposition of cell wall material (Mecchia *et al.*, 2017).

A preponderant role is again played by Ca^{2+} in regulating the release of the male gametes and in fertilization (Iwano *et al.*, 2012). Several molecules initiate their signalling mechanisms and interactions after Ca^{2+} oscillation events. Specific signatures of this secondary messenger are essential in the control of the PT and the PCD of the receptive synergid, in the fusion of the male and female gametes, and hence in the ultimate success of the double-fertilization process (Denninger *et al.*, 2014; Ngo *et al.*, 2014).

Conclusions and outlook

The pathway followed by the pollen tube from the surface of the stigma to the ultimate generation of a new seed is long and varied. The journey encompasses a complex genetic network of regulatory signals and refined cross-talk that provide step-by-step guidance towards the ovule. In this review, we have tried to map this pathway and present it as a series of clear and simple steps, and to highlight where gaps exist in our knowledge.

A broader description of the signalling pathways would certainly include the interactions that exist between the hormonal-signalling control of the positioning of tissue domains and the complex genetic regulatory network of the double-fertilization process, and consider the reciprocal dependency of the pollen tube on the transmitting tissue. A separate review could be written on the roles of Ca^{2+} , which is present during all the stages of the double-fertilization process, and the AGP family, which is responsible for vital interactions at each step. New key players are still being discovered, helping to complete the overall picture of the network of male–female cross-talk during this sexual plant reproduction process.

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