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Review

Signalling between the sexes during pollen tube reception

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Plant reproduction is a complex, highly-coordinated process in which a single, male germ cell grows through the maternal reproductive tissues to reach and fertilise the egg cell. Focussing on *Arabidopsis thaliana*, we review signalling between male and female partners which is important throughout the pollen tube journey, especially during pollen tube reception at the ovule. Numerous receptor kinases and their coreceptors are implicated in signal perception in both the pollen tube and synergid cells at the ovule entrance, and several specific peptide and carbohydrate ligands for these receptors have recently been identified. Clarifying the interplay between these signals and the downstream responses they instigate presents a challenge for future research and may help to illuminate broader principles of plant cell–cell communication.

Highlights

Understanding plant reproduction offers potential routes both to impact on seed crop agriculture and to reveal key principles of plant cell signalling.

Cell–cell signalling during plant reproduction revolves around the interactions between a small family of receptor-like kinases, their coreceptors, and ligands.

Several specific peptides and carbohydrates have recently been confirmed as ligands in reproductive signalling, opening new avenues to investigate their interactions and potentially distinct downstream effects.

Known downstream signalling responses include the production of reactive oxygen species, the generation of specific calcium signatures, and changes to the cell wall.

Fertilisation in flowering plants is a crucial and complex process

Reproduction in flowering plants (**angiosperms**; see Glossary) underlies our agricultural system – without it there would be no seeds or fruits, and therefore no yield from the vast majority of our key crops. Angiosperm **fertilisation** is highly coordinated in space and time. First, the male pollen grain, a single cell containing two male **gametes**, germinates on the female **stigma**. The **pollen tube** emerges by **anisotropic growth** from the pollen grain and grows directionally through the stigma, **style**, and **transmitting tract** of the **carpel** before changing direction to cross the **septum**, grow along the **funiculus**, and approach the **micropylar** end of an **ovule** (Figure 1A–C). Here the pollen tube grows through the **integuments** that surround the female **gametophyte** (or **embryo sac**), temporarily slowing its growth as it reaches the **synergid cells**, presumably to allow cell–cell communication to facilitate pollen tube reception. The pollen tube increases its growth rate as it grows across one of the synergid cells, and eventually bursts to release the enclosed sperm cells which fuse with the **central cell** and **egg cell**, forming the **endosperm** and **embryo**, respectively [1]. Each stage of pollen tube development and fertilisation involves cell-to-cell signalling between the male gametophyte and female tissues. As such, reproduction provides an excellent model system for cell–cell signalling in plants, with the advantage that the genotypes of the two signalling partners can be independently manipulated.

The molecular mechanisms of reproduction and fertilisation have been studied for over 20 years, and FERONIA, the first receptor to be implicated in pollen tube reception at the ovule, was identified in 2003 [2,3]. The past few years have been particularly fruitful in elucidating signalling between the male and female gametophytes. We focus here on the process of pollen tube reception at the ovule in Arabidopsis (*Arabidopsis thaliana*), from the moment of contact between the pollen tube and synergid cell, to pollen tube burst, which releases the sperm cells for **double fertilisation**. We first consider the proteins in the ovular synergid cells that are responsible for detecting signals from the pollen tube, and those at the pollen tube

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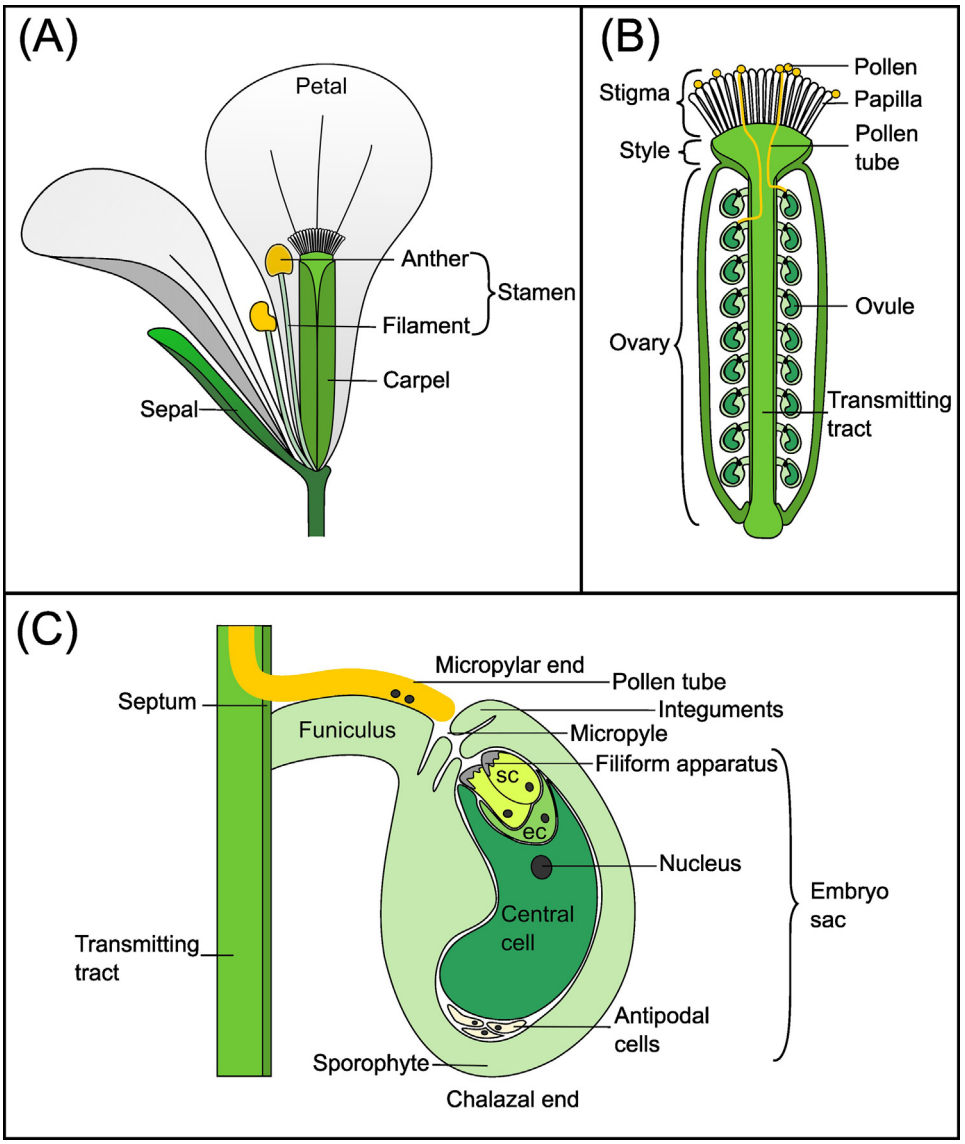

Figure 1. Anatomy of *Arabidopsis thaliana* (Arabidopsis) reproductive tissues. (A) Arabidopsis flower cross-section showing two male stamens, made up of an anther and filament, and the female carpel. (B) Cross-section through the carpel showing the stigma, made up of multiple papillae; the style; and the ovary, containing multiple ovules connected to the septum by funiculi. The male pollen and the path of the pollen tubes are shown. (C) Close-up diagram of the ovule, showing the seven cells which make up the gametophytic embryo sac – the synergid cells with specialised filiform apparatus, the egg cell, central cell, and antipodal cells. The sporophytic tissue including the integuments can be seen surrounding the gametophyte, together with the funiculus which connects the ovule to the septum. The path of the pollen tube across the septum and along the funiculus to the micropyle is shown. Abbreviations: ec, egg cell; sc, synergic cell.

tip that receive reciprocal signals. We then examine the peptide and carbohydrate ligand signals that pass between the pollen tube and ovule. We finally outline our current knowledge of the downstream signalling pathways that are triggered by detection of these ligands.

Glossary

Angiosperms: flowering plants which reproduce by double fertilisation.
Anisotropic growth: growth rates are not equal in all directions.
Antipodal cells: three small, haploid cells at the chalazal end of the ovule. The exact role of these cells is unclear.
Carpel: a female reproductive organ made up of the stigma, style, and ovary. A pistil may comprise a single carpel or multiple fused carpels.
Central cell: the secondary female gamete which contains two fused nuclei, and after fertilisation becomes the endosperm.
Double fertilisation: the process by which the egg cell and central cell of the ovule are each fertilised by the two sperm nuclei from the pollen tube in angiosperms.
Egg cell: the primary female gamete, which after fertilisation becomes the embryo.
Embryo: the initial stage of development of the future seedling. Formed when the female egg cell is fertilised by one of the pollen tube sperm cells.
Embryo sac: the female gametophyte comprising the egg cell, central cell, two synergid cells and three antipodal cells.
Endosperm: a triploid tissue (in diploid Arabidopsis – *Arabidopsis thaliana*) that is formed when the pollen tube sperm nucleus fuses with the double nuclei of the female central cell. A food source for the developing embryo and seedling.
Fertilisation: fusion of male and female gametes that involves both plasmogamy (fusion of cells) and karyogamy (fusion of nuclei).
Filiform apparatus: a highly specialised structure comprising a thickened cell wall with projections into the cytoplasm, and hence increased plasma membrane area, at the micropylar end of the synergid cells, and is involved in communication between the male and female cells.
Funiculus: the stalk that attaches each ovule to the septum.
Gametes: haploid cells containing the parent plant DNA which will fuse to become the offspring, namely that sperm cells and the egg cell.
Gametophyte: the haploid generation of the life cycle of a diploid flowering plant. It comprises the pollen/pollen tube (male) and the embryo sac within the ovule (female).

Anatomy of structures involved in pollen tube reception

Whereas the male gametophyte (pollen grain cell) encases the two sperm cells, the female gametophyte (embryo sac) is multicellular in a more conventional sense, and consists of seven cells – three small **antipodal cells**, the large central cell (which contains two polar nuclei that fuse prior to fertilisation), the egg cell, and two synergid cells at the micropylar end (Figure 1C). It is embedded within maternal **sporophyte** tissue, collectively forming the ovule, which is attached to the septum by the funiculus. The sporophytic tissues surrounding the embryo sac may also regulate reproduction by influencing gametophyte development and maintenance [4,5].

The synergid cells are crucial for signalling during reproduction: initially they attract the pollen tube to grow towards the ovule by secreting cysteine-rich peptide signals (*At*LURE1s, XIUQIU1–4, and TICKETs) ([6–9]; recently reviewed in [10]), after which they interact physically and chemically with the pollen tube throughout its reception. *In vivo* pollen tube burst is closely followed by degeneration of the synergid that received the pollen tube (receptive synergid) [1]. If fertilisation is unsuccessful, the second synergid cell (the persistent synergid) resumes communication with pollen tubes, allowing rescue of failed fertilisation [11]. Once double fertilisation has been completed successfully, secreted pollen tube attractants are actively degraded [12,13] and the persistent synergid cell fuses with the endosperm, terminating attraction of further pollen tubes (**polytubey**) [14].

The role of synergid cells in cell–cell communication is facilitated by the **filiform apparatus** at their micropylar end, a structure with thickened cell wall projections and increased plasma membrane area which allow high rates of signal secretion [15]. Embedded within the filiform apparatus is the pollen tube reception complex, at the core of which sit receptor kinases that bind to peptide and carbohydrate ligands and trigger downstream signalling pathways within the synergids.

Discovery of the synergid-localised pollen tube reception complex

The signals that pass between the gametophytes during pollen tube reception are detected by complexes of receptor kinases and their associated coreceptors. FERONIA (FER) was the first receptor kinase identified as being required for pollen tube reception. Two mutants, *feronia* and *sirène*, with pollen tube overgrowth and polytubey phenotypes were published almost simultaneously [2,3] and later confirmed as allelic [16]. FER is expressed throughout the plant except in mature pollen, and is involved in diverse plant processes across defence and development [16,17]. As a *Cr*RLK1L family receptor kinase, FER has a malectin-like extracellular ligand-binding domain (Box 1), a transmembrane domain, and an intracellular kinase domain. It is highly enriched at the plasma membrane in the filiform apparatus [16].

Coreceptor LORELEI (LRE), a synergid-specific glycosylphosphatidylinositol (GPI)-anchored protein, was next to be implicated in pollen tube reception [18,19]. *lre* mutants displayed striking

Integuments: protective layers of sporophyte tissue surrounding the female gametophyte in the ovule.
Micropyle: the opening of the ovule where the pollen tube enters.
Ovule: the female gametophyte and surrounding maternal tissues. After fertilisation this becomes the seed.
Pollen tube: part of the male gametophyte. It is a tubular extension of the pollen grain that grows toward the female ovule, and eventually releases two enclosed sperm cells to fertilise the egg cell and the central cell.
Polytubey: multiple pollen tubes reach a single ovule, either simultaneously or sequentially, by 'fertilisation recovery' following failure of the first pollen tube to fertilise the ovule.
Septum: the wall of tissue that separates the ovary into separate chambers (locules). The transmitting tract forms between the septum layers, therefore the pollen tube must exit the transmitting tract through the septum to reach an ovule.
Sporophyte: the spore-producing generation of the flowering plant life cycle (the diploid generation in Arabidopsis).
Stigma: the collection of papillae at the top of the carpel which receive the pollen.
Style: the section of the carpel that connects the stigma to the transmitting tract.
Synergid cells: two specialised cells in the egg sac near the micropyle which are involved in signalling between the pollen tube and the female gametophyte.
Transmitting tract: a specialised tissue enclosed by the septum, through which the pollen tube travels to approach the ovules.

Box 1. What is a malectin-like domain?

An important step in our understanding of the *Cr*RLK1L receptors was the discovery of a disaccharide-binding protein in *Xenopus laevis* and recognition that there are similarly folded proteins in plants [62]. The *Xenopus* malectin protein is endoplasmic reticulum-localised and is involved in carbohydrate binding and protein *N*-glycosylation surveillance [62]. A family of plant proteins (mostly receptor kinases) were discovered that contain an extracellular region with homology to the *Xenopus* malectin domain [62]. In Arabidopsis, 13 receptor kinases have a single malectin domain, whereas 62 have a duplicated malectin domain (termed a malectin-like domain) [63]. *Cr*RLK1Ls (first discovered by Schulze-Muth *et al.* [64]) are a subfamily of 17 receptor kinases that contain this extracellular malectin-like domain. They are important in many aspects of plant signalling ranging from plant development to defence [41], and are central to cell–cell communication during pollen tube reception at the synergid cells [65]. A small, related clade of nine receptor-like proteins also have a malectin-like domain, but little is known of their function [63]. Two other similar Arabidopsis proteins have a single malectin domain which is intracellular and attached to a kinesin motor [66]. This variety of malectin domain-containing proteins in plants reflects a diverse range of subcellular localisation patterns and potential functions.

phenotypic similarities to *fer*, and these proteins were later confirmed as direct interactors within a common signalling pathway [20]. LRE has a modified eight-cysteine motif, which is required for pollen tube reception, and an N-terminal signal peptide for transport into the endoplasmic reticulum (ER) [18,19,21]. The GPI anchor is required for LRE localisation to the filiform apparatus plasma membrane but, intriguingly, LRE lacking its GPI anchor is still able to fulfil its function in reproduction [21]. Although one study reported that LRE was required for filiform localisation of FER [20], another reported wild-type FER localisation in the *lre* mutant [22], collectively suggesting that LRE is not essential for FER localisation, but may chaperone FER to the plasma membrane under some conditions. The findings of Liu *et al.* (2016) further support the suggestion that LRE-mediated chaperoning of FER to the synergid plasma membrane is not essential for pollen tube reception because they were able to partially rescue deficiencies in this process in *lre-7* mutants by ectopic expression of a plasma-membrane-targeted LRE in pollen tubes. This intriguing result suggests that sufficient FER to permit pollen tube reception reaches the synergid plasma membrane in the absence of chaperoning by LRE, and indicates a crucial role for LRE at a stage of pollen tube reception when the membranes of the pollen tube and synergid are in sufficiently close contact to allow protein–protein interactions.

Further plasma membrane-associated, GPI-anchored proteins are also involved in pollen tube reception [23]. In contrast to LRE, EARLY NODULIN-LIKE (EN) proteins EN11–EN15 have extracellular plastocyanin-like and arabinogalactan glycomodule domains, suggesting distinct functions. Both quintuple knockdown/knockout lines and EN15 overexpression lines show pollen tube overgrowth, similar to *lre* and *fer*, and EN14 interacts strongly with the extracellular domain of FER, although only weakly with LRE [23]. EN proteins have been proposed to function as adaptors that tether FER to the plasma membrane at the filiform apparatus (Figure 2A) [23].

Two more *Cr*RLK1L receptor kinases, HERCULES RECEPTOR KINASE 1 (HERK1) and ANJEA (ANJ), form complexes with FER and LRE [22]. These proteins act redundantly in pollen tube reception, raising the possibility that complexes may be formed of either HERK1–FER–LRE or ANJ–FER–LRE although, because HERK1 and ANJ are also able to interact with one another, a complex of all four proteins is also plausible [22]. The *fer-4* fertility defect cannot be rescued by expression of the related *Cr*RLK1L receptor kinases HERK1 or ANXUR1 (native to the pollen tube) under the *FER* promoter [24], confirming the importance of having both FER and another *Cr*RLK1L receptor kinase, HERK1 or ANJ, for receptor complex function. If HERK1 and ANJ do indeed form distinct complexes with FER-LRE, this would raise the question of whether they have functionally distinct roles.

Two proteins involved in *N*-glycosylation, EVAN (EVN, a dolichol kinase) and TURAN (TUN, a UDP-glycosyltransferase), have been linked to pollen tube reception through pollen tube overgrowth in heterozygous female *evn* or *tun* mutants (homozygous plants were not recovered) [25]. Because biochemical analysis of *tun* knockdown lines indicates that the pollen tube overgrowth phenotype is not due to lack of glycosylation of FER, *N*-glycosylation via EVN and TUN may regulate other ER-transported members of the pollen tube reception complex such as LRE [25].

A receptor kinase complex at the pollen tube tip

Members of the *Cr*RLK1L receptor kinase family are also found at the pollen tube tip where they receive reciprocal signals from the synergid cells, as well as autocrine signals that maintain the integrity of the pollen tube until cues for burst are received [26]. Four *Cr*RLK1Ls, ANXUR1 and 2 (ANX1/2) [27,28] and BUDDHA'S PAPER SEAL 1 and 2 (BUPS1/2) [26], form heterodimeric complexes that are essential for pollen tube integrity maintenance. ANX1/2 act redundantly,

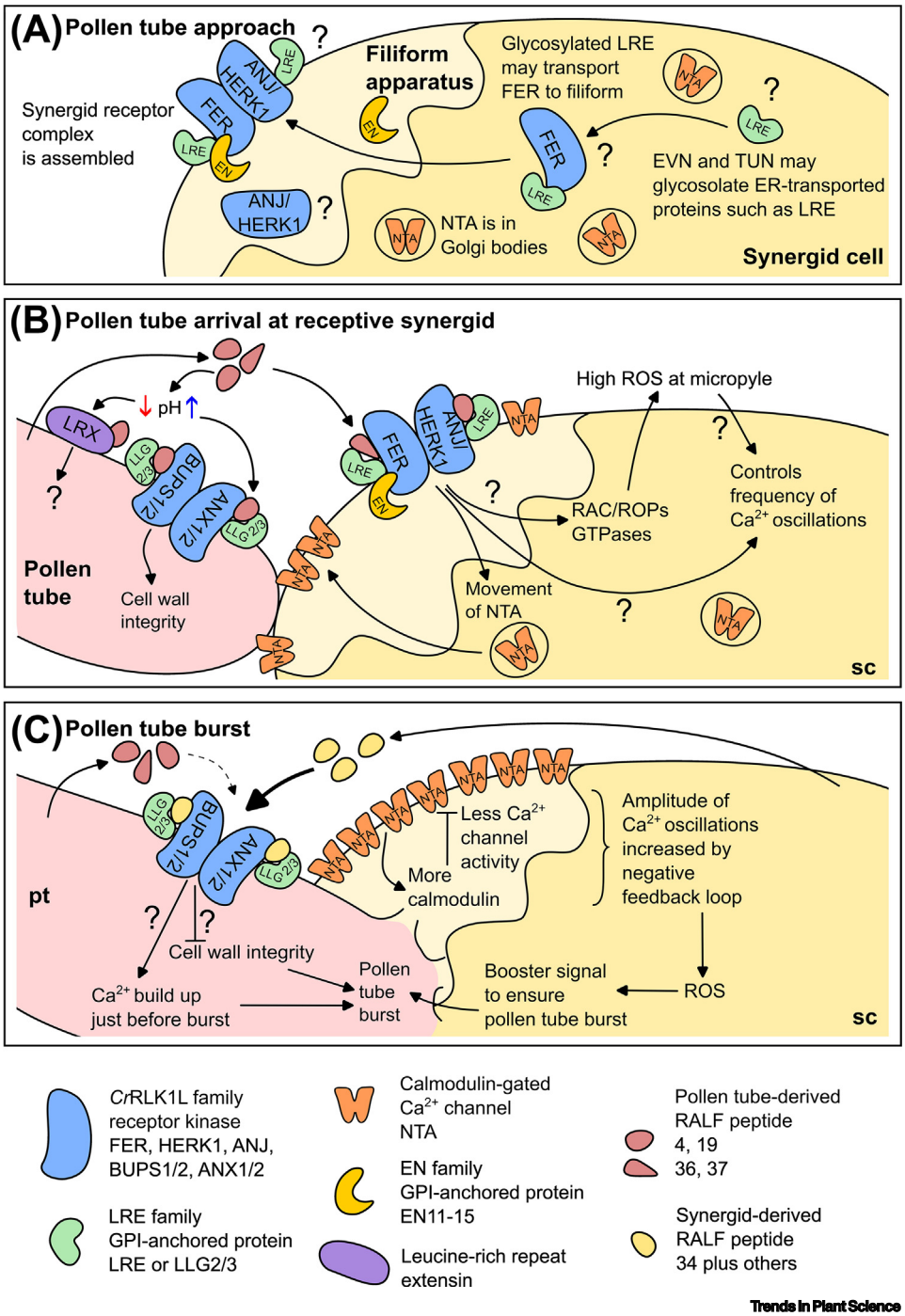

Figure 2. Cell-to-cell signalling during reproduction. (A) The events preceding synergid receptor complex assembly are unclear, but a possible scenario is described. Upon pollen tube approach, LRE may be glycosylated (by e.g., EVN/TUN) then may transport FER to the filiform apparatus, where the FER–HERK1/ANJ–LRE–EN receptor complex is assembled. ANJ and HERK1 may be transported to the filiform apparatus by LRE, or they may already be present in the filiform. NTA is held within Golgi bodies at this stage. (B) Binding of pollen tube-derived RALFs 4/19 is pH-dependent. At low pH, binding to LRXs is enhanced, whereas at high pH RALFs 4/19 preferentially bind to the ANX1/2–BUPS1/2–LLG2/3 receptor complex, thus

(Figure legend continued at the bottom of the next page.)

whereas BUPS1/2 function additively, and BUPS1 is the major player: *anx1/2*, *bups1/2*, and *bups1* mutants all display precocious *in vitro* pollen tube burst of varying severity, as well as failure of pollen tubes to reach the ovules *in vivo* [26–28]. Close homologs of LRE, LORELEI-LIKE GPI-ANCHORED PROTEINS 2 and 3 (LLG2/3), perform its equivalent coreceptor function in the *Cr*RLK1L complex of the pollen tube tip, and chaperone the *Cr*RLK1Ls from the cytoplasm to the plasma membrane [29,30]. Although the role of this complex in maintaining pollen tube cell wall integrity to sustain tip growth has been studied in some detail ([31–33]; recently reviewed in [34]), the changes in signalling during pollen tube reception and burst are less well understood.

Peptide ligands transmit signals between the pollen tube and synergid cells

Small, secreted, cysteine-rich peptides have been proposed as ligands of FER during fertilisation since the identification of the peptide RAPID ALKALIZATION FACTOR 1 (RALF1) as a FER ligand in root growth [35]. There are 37 RALFs encoded in the Arabidopsis genome [36], among other families of secreted cysteine-rich peptides, making identification of the exact peptide(s) involved in any specific signalling pathway challenging.

Ge *et al.* [26] first demonstrated a role for RALFs as ligands that regulate pollen tube integrity and burst *in vitro*, and Mecchia *et al.* [37] simultaneously reported their integrity function. RALF4 and RALF19 from the pollen tube act in autocrine signalling to maintain pollen tube integrity by binding to the ANX1/2–BUPS1/2 complex in the pollen tube tip (Figure 2B). RALF34 is expressed throughout the ovule and, *in vitro*, readily outcompetes RALF4 and RALF19 for binding to the receptor complex to trigger pollen tube burst (Figure 2C) [26]. Despite very low RALF34 concentrations triggering pollen tube burst *in vitro*, *ralf34* mutant plants have no fertility defect, indicating that this peptide is not the only signal capable of inducing pollen tube burst [26].

Two recent publications have particularly advanced our understanding of signalling during reproduction by collectively identifying several specific, pollen tube-expressed RALFs that are perceived by receptor complexes in the synergids. Zhong *et al.* sought to identify proteins involved in the polytubey block at the septum, and found that a subset of the RALFs they identified were also involved in pollen tube reception at the synergid cells [11]. To select candidate genes, they used RNA-seq analysis of a triple *myb* transcription factor mutant with a pollen tube reception defect similar to the *Cr*RLK1L mutants. Pollen tubes of the *ralf36/37* double CRISPR mutant failed to be received by the synergids, causing overgrowth within the embryo sac and implicating this closely related pair of ligands in pollen tube reception. A quintuple CRISPR mutant in which an additional trio of closely related RALFs were also mutated (RALFs 6/7/16) had a more severe pollen tube overgrowth phenotype, although no pollen tube overgrowth was observed in a triple CRISPR line for these three peptides only, suggesting that they are not essential for pollen tube reception, but are able to contribute to this process in the absence of key RALFs 36/37. Interaction of all five of these RALF peptides with receptor kinases FER, ANJ, and HERK1 was confirmed biochemically [11].

maintaining pollen tube cell wall integrity. Downstream effects of the LRX/RALF complex are unknown. When the pollen tube arrives at the receptive synergid, RALFs 4/19/36/37 bind to the FER–HERK1/ANJ–LRE–EN receptor complex, triggering two reactions in the synergid cells: movement of NTA from the Golgi bodies into the filiform apparatus, and accumulation of ROS via interaction with RAC/ROPs which collectively regulate Ca^{2+} oscillations. The ROS response may be ANJ/HERK1-independent. (C) Synergid-derived RALF34 (and likely other RALFs) outcompete RALFs 4/19 for binding site(s) in the ANX1/2–BUPS1/2–LLG2/3 receptor complex, leading to pollen tube burst. Ca^{2+} accumulates in the pollen tube tip before burst. Meanwhile, the magnitude of Ca^{2+} oscillations in the synergids increases via a negative feedback loop where increased Ca^{2+} movement through NTA leads to an increase in calmodulin, thus decreasing NTA Ca^{2+} channel activity. This creates a booster signal to ensure pollen tube burst. Abbreviations: ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; ROS, reactive oxygen species; pt, pollen tube; sc, synergid cell.

The results from Gao *et al*. [38] complement those of Zhong *et al.* [11] because the two studies tested mutually exclusive lists of RALF peptides. Gao *et al.* tested interactions of eight pollen tube-expressed RALFs (selected from [26]) and the ovule-expressed RALF34 for interaction with FER, and concluded that, of these, only RALF4, RALF19, and RALF34 are bound by FER, and that the presence of any of these three RALFs enhances the interaction of FER with the coreceptor LRE [38].

Taken together, these two recent studies show that multiple RALF peptides can act as signals from the pollen tube to the synergid cells during pollen tube reception. Why this functional redundancy is required remains unclear, but it could be to ensure robustness of the response. Our knowledge of signals that pass from the ovule to the pollen tube remains more limited; only RALF34 has been confirmed, and there is evidence that other signals must be involved given the lack of fertility defects in the *ralf34* mutant.

The interactions of *Cr*RLK1Ls with their ligands can be further understood through structural studies (Box 2). Although no *Cr*RLK1Ls have so far been crystallised in complex with reproduction-associated ligands, the structures of unbound receptors and of *Cr*RLK1L–RALF complexes from other plant contexts offer insights into the likely conformation of the receptor kinase complexes found in reproductive tissues.

Carbohydrate monomers as ligands

Because proteins of the *Cr*RLK1L receptor kinase family have an extracellular malectin-like domain, it was anticipated that they would bind carbohydrates like other malectin domain proteins [39,40]. The identification of RALFs as *Cr*RLK1L ligands initially cast some doubt on this hypothesis [17]; however, dual binding specificity for peptides and carbohydrates is now confirmed.

Box 2. The complex 3D puzzle of receptor kinase complexes

No structural analyses of the synergid-expressed FER–LRE–HERK1/ANJ receptor kinase complexes and reproduction-linked ligands have been published to date. However, partial structures of pollen tube *Cr*RLK1Ls, and a full crystal structure of the FER extracellular domain and LLG2 binding a RALF peptide involved in plant immunity, give insights into the possible structure of the reproductive receptor complexes [67,68].

The crystal structures of the extracellular domains of pollen tube-localised ANX1 and ANX2 revealed an unexpected potential binding cleft, located between the tandem malectin-like domains, which may permit interaction with peptide, carbohydrate, and even protein ligands [62,67,69]. Although the *Cr*RLK1L receptors were first considered to be potential carbohydrate-binding proteins because of a region with low homology to the animal malectin proteins (Box 1), evidence from the crystal structures suggests that they may bind carbohydrates via an independent mechanism [67,69]. The crystal structure of the $FER^{extracellular\ domain}$–RALF23–LLG2 complex shows that the association of FER with LLG2 is nucleated by immunity-associated RALF23, which binds to LLG2 via its N-terminus and FER via its C-terminal region, thus providing an interface between the two proteins [68]. The interaction of RALF23 and LLG2 with FER occurs at the membrane-proximal end of the second malectin domain [68], which is physically distanced from the potential binding cleft between the two malectin domains [67,69], and dual binding of carbohydrate and peptide ligands in parallel by FER may therefore be possible.

Although RALF23 is not involved in reproduction, recent evidence shows that other, reproduction-associated RALFs 4/19 enhance the interaction between FER and LRE in pull-down and coimmunoprecipitation (Co-IP) assays [38], suggesting that specific FER–LLG/LRE–RALF complexes assemble in different functional contexts. Xiao *et al.* suggest that RALFs initiate the formation of the receptor complexes, but because FER–LRE interactions have also been detected *in vivo* in the absence of RALFs, the situation *in planta* remains to be clarified [20,38,68,70].

How coreceptors ANJ and HERK1, and proteins of the EN family, fit into a model of RALF binding during pollen tube reception at the ovule remains unknown. It seems likely that RALF binding promotes the interaction of FER with HERK1 or ANJ, as it does with LRE [38]. A swap of the ligand-binding domain of ANX1 into FER was insufficient to complement the *fer-1* mutant [24], suggesting that FER may function uniquely as a scaffold to recruit further receptor kinases, or that *Cr*RLK1L ligand-binding domains have high substrate specificity. Although EN proteins interact with FER *in vitro*, the potential influence of RALFs on this interaction remains to be tested.

*Cr*RLK1L receptors function in cell wall integrity sensing during both development and defence, including regulation of pollen tube maintenance and burst [41].

Feng *et al.* first demonstrated binding of *Cr*RLK1L receptor kinases to carbohydrates *in vitro*. Employing a suite of biochemical techniques, they showed that the extracellular domains of FER, ANX1, ANX2, and BUPSs could interact with polygalacturonic acid (PGA), the building block of pectin [42]. Pectin is one of the most abundant carbohydrates in plant cell walls, and regulates cell wall mechanical properties through changes in its methylesterification status mediated by pectin methylesterase (PME) enzymes and their inhibitors (PMEIs) [43]. BUPS1 and FER have been implicated in both sensing and responding to mechanical stress in pollen tubes and seedlings, respectively [44,45]. This suggests that the dual ligand-binding capability of *Cr*RLK1L receptor complexes allows them to integrate information about cell wall integrity with signals from RALF peptides.

Although molecular interactions between FER and pectin have not been directly studied in the context of reproduction, FER has been indirectly implicated in detecting cell wall changes during fertilisation. Duan *et al.* [12] reported that *fer-4* ovules failed to produce the nitric oxide (NO) burst seen at the filiform apparatus in wild-type plants in response to the application of fragmented PGA *in vitro*, demonstrating that FER is necessary to transduce the response to this cell wall fragment. The authors highlight the distinct roles of FER in ensuring sperm delivery and preventing polytubey, and implicate the PGA-induced NO burst specifically in the latter process by disabling *At*LURE1 peptides.

Most recently, Lin *et al.* [46] corroborated the previous report of pectin binding by the FER extracellular domain *in vitro*, and reported that other cell wall components – cellulose and xylan (a hemicellulose) – are not FER-bound. Furthermore, demethylesterified pectin in the form of PGA was more strongly bound by FER than partially methylesterified pectin. They also demonstrated that FER remains wall-associated rather than plasma membrane-associated after plasmolysis of epidermal pavement cells, supporting the idea that FER binds to the cell wall *in planta*.

These three recent studies consistently indicate a specific role for pectin as a ligand of FER, and demonstrate that pectin signalling through FER contributes to preventing polytubey [12,42,46]. It remains to be seen whether *Cr*RLK1L–pectin interactions contribute to other aspects of reproduction.

Ligand perception in the synergid cells triggers downstream signalling events

Kinase activation of *Cr*RLK1L proteins in their receptor complexes triggers intracellular signalling (Box 3). Seeking downstream components of the FER signalling pathway, Kessler *et al.* identified the synergid-localised NORTIA (NTA) protein, also known as MLO7 (MILDEW RESISTANCE LOCUS O-7) [47]. NTA–GFP relocalises following pollen tube reception from within Golgi bodies to the plasma membrane at the filiform apparatus of both synergid cells [47–49] (Figure 2AB). NTA–GFP relocalisation is not observed in *fer, lre*, or *herk1/anj* mutant plants, confirming dependence on signalling through the pollen tube reception complex [22,47]. Like *fer* mutants, *nta* mutants display pollen tube overgrowth and reduced fertility, although the milder defect in *nta* mutants indicates that NTA cannot be essential for pollen tube burst *in vivo* [47]. Ju *et al.* produced engineered forms of NTA and demonstrated that relocalisation of NTA to the filiform apparatus is necessary and sufficient for fertilisation: Golgi-retained NTA could not rescue fertility in *nta-1*, and filiform apparatus-targeted NTA bypasses the FER signalling complex but complements the reproductive defects of *nta-1* [49]. They propose that signalling from the pollen tube through FER–LRE amplifies calcium signalling at the micropyle and causes NTA relocalisation,

Box 3. Kinase activity of reproduction-linked reception complexes

The intracellular kinase domains of the *Cr*RLK1L proteins are highly conserved in their sequences and may display some redundancy in their roles in propagating ligand signals into cellular responses [24]. Kessler *et al*. demonstrated that kinase domain swaps of FER for the cognate domain of ANX1 or HERK1 complemented the *fer-1* fertility defect [24], indicating functional equivalence between these kinase domains. Removing the kinase activity of FER indicates that FER kinase activity is not required during fertilisation [24,50]. Following the identification of HERK1 and ANJ, and given that their kinase activity is also not required for complementation of the *herk1/anj* double mutant [22], we can hypothesise that the kinase activity of either FER or HERK1/ANJ is sufficient to trigger the downstream cellular responses in pollen tube reception. Whether the kinase activity of BUPS1/2 and/or ANX1/2 is necessary for pollen tube cell wall integrity remains to be tested.

Although FER kinase activity is not explicitly required for downstream responses during pollen tube reception, its kinase activity has been linked to specific lysine residues. Three studies confirmed that the conserved Lys-565 is required for FER kinase activity [24,50,57] based on its position within the active site and the requirement for autophosphorylation during root growth [35]. Together with Lys-663, Lys-565 coordinates ATP positioning within the kinase active site, whereas Lys-699 negatively regulates ATP turnover or binding [71]. Autophosphorylation of Ser-871 and Ser-877 inhibits kinase activity *in vitro*, and *in vivo* regulation of FER by auto- or transphosphorylation of these and other residues possibly contributes *Cr*RLK1L function [71]. Regulation of other pollen tube and synergid *Cr*RLK1L receptors through equivalent mechanisms is anticipated.

which in turn leads to production of a secondary 'booster' signal to ensure that a threshold level of calcium is reached as required for the release of signals to induce pollen tube burst.

The function of NTA and related MLO proteins as calmodulin-gated calcium channels has only recently been discovered [38]. In the COS7 cell system, FER and LRE interact with NTA to promote its relocalisation to the plasma membrane. Although these protein–protein interactions have not been confirmed *in planta*, they are consistent with the lack of NTA relocalisation in *Cr*RLK1L mutants, as described in the preceding text [47–49]. The function of NTA as a calcium influx channel was demonstrated *in planta* by measuring higher synergid calcium levels in cells expressing a version of NTA that was rendered insensitive to negative regulation by mutation of its calmodulin-binding domain, relative to calcium levels in wild-type cells. The kinase activity of FER is not required for normal synergid calcium spiking, consistent with previous reports on pollen tube reception [24,50]. Furthermore, direct plasma membrane targeting of NTA demonstrated that FER and LRE are not required for ion channel functionality [38]. High levels of Ca^{2+} prompt calmodulin binding to NTA which inhibits calcium influx, forming a feedback loop that generates the calcium oscillations in the synergids that are required for pollen tube reception (Figure 2C). In the pollen tube tip, other members of the MLO protein family have been implicated in controlling calcium signalling via a different mechanism – regulating the exocytosis of another, distinct calcium channel [51].

Although Gao *et al.* were the first to report the Ca^{2+} channel activity of NTA and other MLO proteins [38,52], Ca^{2+} signalling was already implicated in pollen tube reception and fertilisation, and distinctive calcium signatures were described in the pollen tube [1], synergids [1,53,54], and female gametes [53,55]. Ngo *et al.* defined four phases of sperm delivery based on the pollen tube and synergid cell calcium signatures and growth behaviours in *semi-in vitro cum septum* live imaging experiments, making detailed comparisons between wild-type, *fer*, *lre*, and *nta* ovules [1]. Their observations suggested that the FER–LRE complex is necessary to initiate essential, typical calcium oscillations in the synergids, whereas NTA modulates the magnitude of calcium oscillations (Figure 2B,C). They also reported that calcium concentration in the pollen tube tip is highly elevated during the second, rapid growth phase across the receptive synergid, and peaks at the point of pollen tube burst [1].

Independently of the NTA pathway, reactive oxygen species (ROS) production also acts between FER and Ca^{2+} signalling to influence pollen tube burst: FER regulates production of high ROS at

the micropyle *in vitro*, which is required for pollen tube burst [56]. Exogenous application of ROS also induces pollen tube burst [54]. HERK1 and ANJ are not required for micropylar ROS accumulation, whereas the requirement for LRE is subject to debate [22,56]. ROS are generated by NADPH oxidase enzymes, and the role of RAC/ROP GTPases in regulating plant ROS production is well established [57]. FER has been shown to signal via RAC/ROPs in various cellular contexts including root hair growth [58] and pavement cell morphogenesis [46,59]. It seems likely that an analogous pathway generates ROS in the synergids, and the reported interaction of synergid-specific LRE as well as FER with ROP2 provides an initial clue to potential specific players [20,56].

Pollen tube growth rate changes and eventual burst, and receptive synergid degradation, must be controlled by careful regulation of the relationship between intracellular turgor pressure and cell wall mechanical strength, the specifics of which are poorly understood. Four cell wall-localised, plasma membrane-associated extensin proteins (LEUCINE-RICH REPEAT EXTENSINS 8–11, LRX8–11) have been implicated in the process of tip growth in the pollen tube [37,60], and may therefore also contribute to determining the timing of pollen tube burst *in planta*. They influence cell wall composition and mechanical properties indirectly through their effects on the release and/or integration of new cell wall material from exocytic vesicles directed to the rapidly growing pollen tube tip [60]. Intriguingly, pollen-expressed LRXs have been shown to interact with RALFs 4/19, indicating that these RALFs can influence pollen tube growth through a second signalling pathway that is independent of the RALF–LLG–ANX–BUPS complex. The balance of RALF binding to LXR or LLG receptors is regulated by pH, and low pH favours LRX binding [61]. Thus RALFs that are folded and oxidised at low pH (pH 5) may bind to oligomeric extensins via their C-terminal ends to regulate cell wall integrity through that pathway, whereas at higher pH (pH7.5; via changes in redox state and cell wall pH) RALF peptides may become reduced and linearised, and extensins may become monomeric, which then promotes binding of RALFs to *Cr*RLK1L/LLGs via their N-terminal ends [61].

A role for pectin in the synergid cells in pollen tube reception has also been suggested. FER may help to maintain demethylesterified pectin at the filiform apparatus because detection of this pectin type was reduced in *fer-4* mutants [12]. Lines with elevated pectin methylesterification (*pme34*, *pme44*, and PMEI1-overexpressor) also show elevated rates of polytubey in common with the *fer-4* mutant [12], although more recent work suggests that polytubey is regulated at the septum, before the pollen tubes reach the synergids [11], and filiform-localised pectin may therefore have a distinct function. Because pectin and its constituents can be both upstream ligands and downstream targets of FER, this raises the possibility of feedback loops [12].

Concluding remarks and future perspectives

Communication between the pollen tube and synergid cells during pollen tube reception is a complex process that involves both peptide and carbohydrate ligands interacting with receptor complexes to generate gaseous and calcium-based downstream signals, which ultimately control pollen tube growth rate, growth direction, and burst. Recent improvements in our understanding of the role of pollen tube-derived RALF ligands represent a major step forward in identifying specific molecular players, but open new questions regarding the reasons for the high apparent redundancy among these proteins, and the potential roles of further RALFs, particularly as reciprocal signals from the ovule (see Outstanding questions). The multitude of proteins implicated in the synergid-localised pollen tube reception complex also present challenges for understanding the 3D receptor complex structures and how they are assembled.

Outstanding questions

What other ovule-derived signals promote pollen tube burst? RALF34 signals from the ovule to the pollen tube, but pollen tube burst is not impaired in the *ralf34* mutant, indicating that other ligands must be involved.

What is the role for LRE in chaperoning CrRLK1L receptor kinases and their coreceptors? Mixed results have been reported concerning the role of LRE in the proper localisation of FER in the synergids, and for ROS production in the micropyle. Is the requirement for LRE in these processes dependent on environmental or intracellular conditions? Do HERK1 and ANJ also require chaperones for their correct localisation, or coreceptors for their functions?

How do the *Cr*RLK1L receptor kinase complexes integrate signals from cell walls with signals from RALF peptides? Do the two types of ligand compete for binding to the receptor complex, or can they bind simultaneously? Do specific conditions favour the binding of each ligand type?

How do RALF peptides integrate signalling between *Cr*RLK1L receptor kinases/LREs and the LRX extensins during pollen tube burst? How do intercellular and/or apoplastic conditions, such as pH and ROS levels, impact on the balance between binding of RALFs to their alternative receptors – the *Cr*RLK1Ls and the LRXs? How do these local environmental conditions vary over time, and does RALF binding feed back directly to influence them?

What is the physical mechanism of pollen tube burst? How does the delicate interplay between pollen tube cell wall strength and cellular turgor pressure transition from a state of balance that maintains growth to an imbalance that causes cell rupture?

Signalling between the pollen tube and the synergid cells is a dynamic and evolving field of research that has implications for plant reproduction and agriculture. As researchers continue to delve deeper into the receptors and ligands involved, and the downstream events, we can expect new insights which may help in understanding the vulnerability of reproduction to environmental conditions, as well as the wider rules of cell–cell signalling in plants.

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Declaration of interests

The authors declare no conflicts of interest.

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