The role of diverse LURE-type cysteine-rich peptides as signaling molecules in plant reproduction

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Abstract

In angiosperm sexual reproduction, the male pollen tube undergoes a series of interactions with female tissues. For efficient growth and precise guidance, the pollen tube perceives extracellular ligands. In recent decades, various types of secreted cysteine-rich peptides (CRPs) have been identified as peptide ligands that regulate diverse angiosperm reproduction processes, including pollen tube germination, growth, guidance, and rupture. Notably, in two distant core eudicot plants, multiple LURE-type CRPs were found to be secreted from egg-accompanying synergid cells, and these CRPs act as a cocktail of pollen tube attractants for the final step of pollen tube guidance. LURE-type CRPs have species-preferential activity, even among close relatives, and exhibit remarkably divergent molecular evolution with conserved cysteine frameworks, demonstrating that they play a key role in species recognition in pollen tube guidance. In this review, I focus on “reproductive CRPs,” particularly LURE-type CRPs, which underlie common but species-specific mechanisms in angiosperm sexual reproduction, and discuss their action, functional regulation, receptors, and evolution.

Keywords

Pollen tube growth; Pollen tube guidance; Synergid cell; Cysteine-rich peptide (CRP); Attractant; Receptor kinase

1. Introduction

Successful sexual reproduction in multicellular organisms requires a series of male-female interactions. In flowering plants (angiosperms), pollen grains germinate and form a pollen tube after pollination on the pistil's stigma (Fig. 1). The pollen tube, a tip-growing male gametophyte carrying immotile sperm cells, grows into the transmitting tissue of the style and enters the ovary, where the ovules are located [1,2]. In the ovary, the pollen tube is controlled by female tissues to precisely target the embryo sac (female gametophyte) of the ovule, known as pollen tube guidance [3,4]. Pollen tube guidance controls the direction and efficiency of the pollen tube tip growth as well as one-to-one pollen tube attraction to the ovule via spatiotemporal and species-preferential male-female communication, which maximizes the interaction rate between male (sperm cells) and female gametes (egg and central cells) inside the ovule [5–7].

In addition to small chemical compounds, plants also use various small secreted peptides for specific intercellular communication events involved in plant growth and development, environmental responses, defense against pathogens, and symbiosis [8]. Plant-secreted peptides include two major types: post-translationally modified peptides and cysteine-rich peptides/polypeptides (CRPs) [9,10]. While most post-translationally modified peptides and proteolytically processed types of CRPs, such as epidermal patterning factor (EPF) and rapid alkalinization factor (RALF) peptide families, have been reported to be involved in various cellular processes in vegetative tissues, several CRPs have been proposed to have specific functions for successful sexual reproduction in angiosperms [11,12].

This review focuses on “reproductive CRPs” that function as signaling

molecules/ligands involved in various aspects of angiosperm sexual reproduction. In particular, LURE-type CRPs, which include multiple pollen tube attractant peptides secreted from egg-accompanying synergid cells, are highlighted as key peptide ligands for species-preferential pollen tube guidance toward the embryo sac. Based on the identification, functional regulation, receptors, and structure of LURE peptides, I discuss how angiosperms accomplish reproduction using a cocktail of attractant peptides.

2. Diverse CRPs as signaling molecules in plant reproduction

CRPs are small secreted peptides/polypeptides (approximately 20–100 amino acids in length) that are encoded by a large gene family (more than 825 genes in *Arabidopsis thaliana* and 598 genes in *Oryza sativa*) and exhibit a relatively rapid molecular evolution, including sequence divergence and lineage-specific gene multiplication [13,14]. Plant CRPs include defensin-like proteins (DEFL), which are thought to have evolved from antimicrobial peptides, and plant-specific classes, such as lipid-transfer proteins (LTP), thionins, and RALFs. While cysteine patterns of CRPs are conserved within subgroups due to the formation of intramolecular disulfide bridges that stabilize three-dimensional folding [15], primary amino acid sequences tend to be highly variable. Interestingly, numerous CRP members from each subgroup are characteristically expressed in reproductive tissues [16–18]. The actual functions of many CRPs have been reported as extracellular signaling molecules or ligands that control various reproductive processes, including pollen tube germination, growth, attraction, rupture, and gamete activation (Fig. 1).

2.1. CRPs involved in pollen tube germination and growth

Upon pollination of Brassicaceae, S-locus cysteine-rich/S-locus protein 11 (SCR/SP11), an ~6 kDa DEFL peptide with eight cysteines, acts as a male determinant of the self-incompatibility response, inhibiting the hydration and growth of self-pollen on the stigma [19,20]. SCR/SP11 peptides are highly polymorphic among the haplotypes and are specifically recognized by an S receptor kinase (SRK) containing an S-locus glycoprotein (SLG)-like ectodomain, which is similarly polymorphic as SCR/SP11 peptides, to induce intracellular signaling in papilla cells [21–24]. In the poppy (*Papaver rhoeas*), a secreted stigmatic S-protein with four conserved cysteines, named PrsS (*P. rhoeas* stigma S), triggers a self-incompatibility response through interaction with the highly polymorphic transmembrane protein PrpS (*P. rhoeas* pollen S) [25–27]. These examples of ligand-receptor pairs, SCR/SP11-SRK and PrsS-PrpS, highlight the functional utility of certain CRP ligands with highly variable small peptide sequences and regulate specific cellular events in male-female recognition.

For efficient and continuous pollen tube growth in the stigma, style, and transmitting tissue of the pistil, the pollen tube receives extracellular ligands secreted from the male pollen and female tissues. In tomato, LAT52, an ~18 kDa potentially glycosylated polypeptide belonging to the Ole e I subgroup, likely regulates pollen hydration *in vitro* and pollen tube growth in the style [28]. The *LAT52* gene is abundantly and specifically expressed in pollen, as its promoter is generally used as pollen-specific overexpression promoters for various angiosperm species. LAT52 interacts with LePRK2, which is one of pollen-specific receptor kinase (PRK) family receptors, expressed in the mature pollen of tomato [29]. Therefore, it has been suggested that LAT52 acts as an autocrine ligand that controls pollen germination and

the early stage of pollen tube growth. As ligands for autocrine signaling in the pollen tube, RALF peptides (RALF4 and RALF19) control pollen tube integrity by interacting with pollen tube surface receptor complexes including ANX1/2 and BUPS1/2, which are *Catharanthus roseus* RLK1-like (CrRLK1L) family receptors, and leucine-rich repeat extensin (LRX) family proteins [30–33]. Since pollen tube integrity modulated via RALF4/19 signaling is also required for continuous pollen tube growth and fertilization, multiple sets of autocrine peptides, as well as other CRP ligands from female tissues, coordinately modulate the growth efficiency and integrity of the pollen tube.

Tomato STIGMA-SPECIFIC PROTEIN1 (LeSTIG1), a secreted polypeptide with a conserved C-terminal cysteine-rich domain that seems to be cleaved in the stigma exudate to generate a ~7 kDa mature peptide, has also been identified as a ligand for LePRK2 [34,35]. It has been proposed that LeSTIG1 from the stigma replaces LAT52 binding to LePRK2 after pollen germination [34]. The C-terminal cysteine-rich domain, but not the N-terminal portion of LeSTIG1, showed activity in stimulating pollen tube growth that was partially dependent upon LePRK2 [35]. Interestingly, the STIG1 ortholog in *A. thaliana*, named GRIM REAPER (GRI), has been identified as a factor involved in reactive oxygen species-mediated cell death, despite the N-terminal portions of LeSTIG1 and GRI being poorly conserved [36]. In contrast to LeSTIG1, the N-terminal fragment of GRI cleaved by a metacaspase (AtMC9) has the activity and binds to PRK5, which is one of eight PRK family receptors of *A. thaliana* [36,37]. Although the detailed function of GRI peptide in reproduction has been unexplored, the *gri* mutant has a reduced seed set [38], suggesting a similar role to LeSTIG1 in the pistil.

2.2. CRPs involved in pollen tube rupture and double fertilization

After the pollen tube reaches the synergid cell, pollen tube rupture is required to release the sperm cells into the embryo sac. A process called pollen tube reception, which is involved in pollen tube rupture at the synergid cell, has been intensively studied as one of the key steps for species recognition in the sexual reproduction of angiosperms [2]. In maize, *Zea mays* embryo sac 4 (ZmES4), a synergid-expressed DEFL, possesses the species-preferential activity required to rupture the pollen tube, possibly via the opening of the potassium channel KZM1 [39]. Interestingly, a pollen-expressed CrRLK1L family receptor, encoded by *Ruptured Pollen tube* (*RUPO*), interacts with the potassium channel HAK1 to control pollen tube integrity in rice [40]. RUPO is phylogenetically related to *A. thaliana* BUPS1/2 rather than ANX1/2. Although synergid-derived ligands that induce pollen tube rupture remain unknown in *A. thaliana*, RALF34 expressed in the ovule, but not in the embryo sac, triggers pollen tube rupture *in vitro* and competes with RALF4/19 to bind with BUPS and ANX receptors [30]. Therefore, the modulation of RALF-CrRLK1 signaling in the pollen tube could be a mechanism involved in pollen tube reception. However, this should be investigated by identifying a required set of peptides and direct monitoring of physiological and molecular dynamics during pollen tube rupture at the synergid cell.

Pollen tube growth through the pistil and the expression of three closely related MYB transcription factors, MYB97, MYB101, and MYB120, are required to induce pollen tube competency, ensuring proper rupture at the synergid cell [41,42]. Downstream of the MYB transcription factors in the pollen tube, over 20 genes encoding thionin peptides of the CRP2460 subgroup are expressed [43]. Interestingly,

these pollen-expressed thionin peptides exhibit rapid molecular divergence, including lineage-specific gene expansion and increased polymorphism rates. Therefore, they may play a key role in pollen tube reception through, for example, the induction of synergid cell burst.

In angiosperms, sperm cells released from the pollen tube undergo double fertilization. Egg-secreted EGG CELL1 (EC1) peptides belonging to the LTP subfamily activate sperm cells for membrane fusion with the egg and central cells [44]. Remarkably, an egg cell of the basal angiosperm species *Amborella trichopoda*, a sister species to all other extant angiosperms, expresses a gene encoding the EC1 ortholog [45], implying the functional conservation of the EC1 peptide to control double fertilization in angiosperms.

3. LURE-type CRPs: a cocktail of pollen tube attractants from the synergid cell

3.1. Identification of pollen tube attractants (LURE peptides) in *Torenia*

Over a century ago, it was thought that the ovule diffuses attraction signals, as it was discovered that pollen tubes grow toward excised ovules on media [46–48]. More recently, a study using laser cell ablation combined with a semi-*in vitro* pollen tube guidance assay of the eudicot *Torenia fournieri* demonstrated that the two synergid cells adjacent to the egg cell are the source of attractants [49]. Furthermore, attraction by synergid cells exhibits species-preferential activity among closely related species, suggesting that attractants are not completely different but can can be rapidly evolving molecules, such as peptides/polypeptides [50].

At least 16 CRPs were identified in 256 contigs constructed from expressed sequence tags of isolated *T. fournieri* synergid cells, and TfCRP1 and TfCRP3 have

been identified as the pollen tube attractants, referred to as LURE1 and LURE2 in *T. fournieri* (TfLURE1 and TfLURE2) [51]. TfLURE1 and TfLURE2 are secreted peptides (with 62 and 70 amino acids in mature peptides, respectively) belonging to the DEFL subfamily with six cysteines, which are abundantly and specifically expressed in synergid cells. In a semi-*in vitro* condition that activates pollen tube competency required to sense the attraction signal from *T. fournieri* ovules [52], *E. coli*-expressed recombinant TfLURE1 and TfLURE2 showed attraction activity toward *T. fournieri* pollen tubes. The downregulation of either *TfLURE1* or *TfLURE2* by injecting morpholino antisense oligos decreased the rate of ovules attracting pollen tubes on the medium. Therefore, TfLURE1 and TfLURE2 have been proven to play major roles in pollen tube attraction by the synergid cells in *T. fournieri*. However, there are other synergid-expressed CRPs, including the same type of DEFLs, namely LURE-type CPRs or CRP810 peptides [10,11,13]. In the close relative *Torenia concolor*, TcCRP1, an ortholog of TfLURE1 (TfCRP1), has also been shown to be a synergid-expressed attractant [53]. TfLURE1 and TcCRP1 exhibit high percentages of attraction to *T. fournieri* and *T. concolor* pollen tubes, respectively, implying that LURE1 peptides of the two *Torenia* species act as key molecules that impart species-preferentiality in pollen tube attraction toward synergid cells.

3.2. Identification of a species-specific cluster of LURE peptides in *Arabidopsis*

In *A. thaliana*, *MYB98*, which encodes a transcription factor specifically expressed in the synergid cells, is essential for pollen tube guidance toward the ovule [54]. In combination with differential expression screenings identifying female gametophyte-expressed genes, this finding led to the identification of many CRPs that

199 are specifically expressed in the synergid cells [16,17,55], prompting researchers to
200 explore CRP attractants derived from *A. thaliana* synergid cells. Through a
201 genome-wide survey of characteristically multiplied DEFLs, Takeuchi and Higashiyama
202 demonstrated that *DEFL* genes, which are expressed in the synergid cells and form a
203 species-specific gene cluster, encode pollen tube attractants of *A. thaliana* (AtLURE1
204 peptides) [56]. AtLURE1 peptides are functional homologs of *Torenia* LUREs, but are
205 phylogenetically unrelated to *Torenia* LURE1. Nevertheless, AtLURE1 and *Torenia*
206 LUREs belong to CRP810 peptides but show little sequence similarity, except for a
207 six-cysteine pattern. In *A. thaliana* Col-0 accession, *AtLURE1* consists of five tandemly
208 arrayed genes (*AtLURE1.2-1.6*) and one gene (*AtLURE1.1*) at a small distance
209 (approximately 100 kb) in the same chromosome. The AtLURE1.5 peptide, which lacks
210 a conserved cysteine, has no attraction activity and the *AtLURE1.6* gene contains a stop
211 mutation in the Col-0 accession, whereas the amino acid sequences of AtLURE1
212 (AtLURE1.1–1.5) are highly homologous (80–95% amino acid sequence identity).
213 Although tandem and segmental gene multiplication events are evolution
214 patterns characteristically observed in *CRP* genes of *A. thaliana* and *O. sativa* [14],
215 *AtLURE1* gene multiplication appears striking. Phylogenetic analysis indicated that
216 *AtLURE1* genes form a species-specific cluster that is separated from a cluster of
217 orthologous genes of the closest relative *Arabidopsis lyrata LURE1* (*AlLURE1.1–1.10*)
218 ([56]; Fig. 2). This species-specific gene multiplication of *Arabidopsis LURE1* might
219 cause functional redundancy; moreover, it might contribute to increased expression and
220 secretion of AtLURE1 or AlLURE1 as a cocktail of attractants and ensure
221 species-preferential pollen tube guidance. In fact, all functional AtLURE1 peptides
222 (AtLURE1.1, AtLURE1.2, AtLURE1.3, and AtLURE1.4) bind to the pollen tube

receptor PRK6 [57].

3.3. Pollen tube attraction by a cocktail of multiple CRP810 attractants

Eight additional *CRP810* genes (*CRP810_2.1*, *CRP810_2.2*, *CRP810_2.3*, *CRP810_3.1*, *CRP810_3.2*, *CRP810_4*, *CRP810_5*, and *CRP810_6*) are expressed in the pistil and downstream of the synergid-specific MYB98, implying that they function as attractant peptides in *A. thaliana* [16,55,56]. Two independent studies have demonstrated that most of these CRP810 peptides have pollen tube attraction activity [58,59]. Since CRP810_3.1 and CRP810_3.2, the closest relatives to the AtLURE1 cluster, showed a species-specific attraction activity to *A. thaliana* and dependence upon PRK6, they were named AtLURE1.7 and AtLURE1.8 [58]. This second cluster of AtLURE1 peptides, namely CRP810_3.1/AtLURE1.7 and CRP810_3.2/AtLURE1.8, appeared to work with the first cluster of AtLURE1 peptides for effective guidance of *A. thaliana* pollen tubes toward the ovule in the *A. thaliana* pistil. Interestingly, I identified 14 genes encoding orthologous *A. lyrata* CRP810_3 (Fig. 2). The two sets of *At/AlLURE1* genes being species-specifically copied within the two *Arabidopsis* species suggests the presence of synergid-secreted attractants that guarantee preferential rather than completely specific attraction of conspecific pollen tubes.

Among other *A. thaliana* CRP810 peptides, CRP810_4/XIUQIU1, CRP810_5/XIUQIU2, and CRP810_2.3/XIUQIU4/TICKET3 showed non-species-preferential attraction to *A. thaliana* and *A. lyrata* pollen tubes, whereas CRP810_2.2/TICKET2 showed *A. thaliana*-specific attraction [58,59]. A TICKET ortholog in each of *A. lyrata* and *Capsella rubella* (AlTICKET and CrTICKET) also showed attraction activity to the conspecific pollen tube [59]. Although *E. coli*- or insect

247 cell-expressed recombinant AtLURE1.2, a representative of the first AtLURE1 cluster,
248 exhibited obvious attraction activity (nearly 100% at a concentration of less than 5 μM)
249 according to three research groups [56,58–61], other CRP810 peptides exhibited a lower
250 activity (~60% at a concentration of 50 μM in the case of XIUQIU1, which showed the
251 highest percentage among these peptides) than AtLURE1.2. Despite this distinct activity
252 and a high expression of the first cluster of AtLURE1 peptides, RNAi-knockdown
253 exhibited a slight defect in pollen tube guidance around the micropyle (the entrance to
254 the embryo sac of the ovule) in the pistil [56]. Furthermore, *atlure1* null (a septuple
255 mutant for *AtLURE1.1-1.5*, *CRP810_3.1/AtLURE1.7*, and *CRP810_3.2/AtLURE1.8*
256 genes) and *ticket1/2/3* (a triple mutant for three *CRP810_2* genes), which were
257 generated by the CRISPR/Cas9 system, still retained normal fertility but exhibited a
258 slight reduction in pollen tube targeting to the ovules at an earlier time after pollination
259 [58,59]. Finally, efforts to generate a hendecuple knockout mutant for the two sets of
260 *AtLURE1* plus *XIUQIU1-4* have revealed that these CRP810 peptides contribute to
261 fertility, despite a reduction of only ~20%, by controlling pollen tube targeting to the
262 ovules in the pistil [58].

263 Collectively, two distant eudicot species, *T. fournieri* (asterids) and *A. thaliana*
264 (rosids), and their relatives utilize synergid-secreted DEFLs (LURE-type CRP810
265 subfamily peptides) as a cocktail of multiple pollen tube attractants. This implies that
266 CRP peptides with a similar cysteine framework are commonly used as ligands to attract
267 conspecific pollen tubes in core eudicots, whereas a secreted non-CRP (ZmEA1) has
268 been identified as an attractant of the monocot maize [62,63]. Because *A. thaliana*
269 pistils lacking most *CRP810* genes still retained good performances in pollen tube
270 guidance and fertility, there should be additional guidance cues. They presumably

include additional CRPs and/or small chemicals derived from the synergid cell and other female cells, as well as mechanical guidance mechanisms, such as pollen tube adhesion on the ovule surface. Further identification of guidance cues and understanding of their divergence in various angiosperm species should clarify how angiosperm females achieve rendezvous with male pollen tubes in a species-specific manner.

4. Functional regulation and recognition of LURE peptides

4.1. Regulation of *LURE* gene expression in synergid cells

Approximately 50 *CRP* genes, mainly from two subfamilies [*DEFLs* (*CRP810*) and *LTPs* (*CRP3700*, *CRP3730*, and *CRP3740*)] are regulated downstream of the MYB98 transcription factor in *A. thaliana*, and most of their promoters have a common cis-element (GTAACNT) containing the *in vitro* MYB98-binding sequence TAAC [55,64]. While 11 *CRP3700* genes are likely to be direct targets of MYB98, five *CRP810* (*AtLURE1.1–1.5*) genes are suggested to be indirect downstream targets of MYB98 because of the requirement of another cis-element (AACGT) instead of GTAACNT [64]. Most of the other *CRP810* genes of *A. thaliana* and *TfLURE2* of *T. fournieri* also possess the AACGT element within 200-bp upstream of the ATG codon (data not shown; [56]). Interestingly, *CRP810_2.1/TICKET1* and *CRP810_6/XIUQIU3*, which are expressed in synergid cells but encode peptides lacking attraction activity, do not possess the AACGT element. It is important to identify factors directly mediating transcription through the cis-element AACGT and understand how the attractant genes are integratedly regulated for synergid-specific expression.

Laser cell ablation in the mature embryo sac of *T. fournieri* has shown that

neither the egg nor central cells are required for pollen tube attraction [49]. However, when either the egg or central cell within immature embryo sacs was disrupted, followed by *in vitro* ovule culturing, ovules with morphologically normal synergid cells exhibited a reduction in attraction activity [65]. After the disruption of an immature egg cell, expression of *TfLURE2*, as a synergid cell specification marker, decreased in one of two synergid cells during embryo sac maturation. Similarly, *A. thaliana* mutants for *CENTRAL CELL GUIDANCE* (*CCG*) and *CCG BINDING PROTEIN1* (*CBP1*), which encode transcriptional regulators in the central cell, showed defects in pollen tube guidance around the ovule and in AtLURE1 production from the synergid cells [56,66,67]. These studies indicate that communication between the egg-synergid and central-synergid is required for acquiring synergid cell function and thus restricts strong attraction by immature embryo sacs.

4.2. Regulation of LURE peptide secretion from synergid cells

Synergid cells are highly polarized, with a characteristic cell wall structure at their micropylar region, a filiform apparatus, and secretion activity toward the micropylar end of the ovule to attract and receive the pollen tube [54,68]. Previous studies have highlighted the accumulation of LURE peptides, other CRP810 peptides, and the maize attractant ZmEA1 around the filiform apparatus [51,56,58,59,62]. This directed localization is important for generating strong attraction signals to guide pollen tubes from some distance. Here, by generating and observing each CRP810 peptide fused with a yellow fluorescent protein (Citrine), driven by each native promoter, I reinvestigated whether each CRP810 peptide showed slightly different localization/diffusion patterns. Consistent with previously reported directed localization

[56,58,59], most CRP810 peptides, except for CRP810_2.1/TICKET1 and CRP810_2.2/TICKET2, were localized at the micropylar end of the synergid cells in unfertilized mature ovules (Fig. 3). However, in the ovules expressing fusion proteins for AtLURE1.2, CRP810_3.1/AtLURE1.7, CRP810_3.2/AtLURE1.8, or CRP810_4/XIUQIU1, diffused fluorescent signals were further detected along the surface of the ovule integument cells around the filiform apparatus toward the micropyle (Fig. 3). The diffusion patterns of these proteins appeared to exhibit slight but substantial differences. Despite being fluorescent fusion proteins, their different diffusion patterns may reflect the functional variation of CRP810 peptides, which could work at different distances from the synergid cells [56,58,59]. LURE peptides have been proposed as guidance cues of a short distance, i.e., 100–150 μm from the micropyle, corresponding to the attraction range in semi-*in vitro* guidance assays of both *T. fournieri* and *A. thaliana* [51,52,56,69]. However, the actual effective distance *in vivo*/in the pistil remains unclear. Therefore, it is important to investigate the spatial and temporal regulation of the directed secretion and diffusion of each CRP810 peptide in the pistil.

Pollen tube attraction by synergid cells should be temporally regulated to achieve a one-to-one relationship between the pollen tube and embryo sac. As noted above, the secretion of attractant CRP810 peptides is likely to be regulated independently of pollination. More specifically, live-cell imaging of embryo sac development in the *in vitro* ovule culture system demonstrated that the expression of *LURE* genes (*TfLURE2* and *AtLURE1.2* in *T. fournieri* and *A. thaliana*, respectively) was initiated during synergid cell maturation after cellularization [65,70]. However, *A. thaliana* octuple mutants for all *ACS* (1-aminocyclopropane-1-carboxylic acid (ACC)

343 SYNTHASE) genes had a decreased proportion of ovules with AtLURE1.2-GFP
344 localization in the filiform apparatus [71]. ACC is a precursor of ethylene, a
345 phytohormone. AtLURE1.2-GFP localization at the filiform apparatus was restored by
346 ACC treatment, but not by ethylene, probably through the GLUTAMATE
347 RECEPTOR-LIKE (GLR)-mediated Ca^{2+} elevation in the ovule. Therefore, there should
348 be a regulatory mechanism for the initiation of attractant secretion. In the future, it will
349 be interesting to investigate how the ACC-induced Ca^{2+} elevation in the ovule activates
350 the secretion of AtLURE1.2 and other CRP810 peptides from synergid cells.

351 After the arrival and rupture of the first pollen tube in one of two synergid cells,
352 successful fertilization with both the egg and central cell is required to prevent
353 secondary pollen tube attraction by the other synergid cell, the persistent synergid cell
354 [72]. Egg cell fertilization likely induces nuclear degeneration of the persistent synergid
355 cell via ethylene signaling, whereas central cell fertilization triggers the
356 synergid-endosperm fusion that leads to the dilution of the contents of the persistent
357 synergid cell [73,74]. These two controls appear to robustly eliminate the residual
358 function of the persistent synergid cell and rapidly inactivate AtLURE1 secretion [74].
359 The CrRLK1L family receptor FERONIA (FER) was originally identified as a key
360 factor in controlling pollen tube reception in synergid cells [75]. The *fer* mutant ovule
361 shows multiple pollen tube attraction, which was assumed to be due to a failure to
362 induce pollen tube rupture and is similar to a phenomenon termed “fertilization
363 recovery” observed in ovules accepting fertilization-defective mutant pollen tubes
364 [72,76–78]. However, it has been demonstrated that FER is involved in a blocking
365 mechanism of supernumerary pollen tubes earlier than the fertilization recovery
366 mechanism and regulates de-esterified pectin and nitric oxide (NO) accumulation in the

filiform apparatus [79]. Additionally, the treatment of ovules with compounds generating NO reduced the AtLURE1.2-GFP localization in the filiform apparatus. *In vitro*, NO induced nitrosation of cysteine residues Cys17 and Cys84 of AtLURE1.2. Cys17 is located in a signal peptide and conserved among most CRP810 attractants. A mutant AtLURE1.2, with alanine substitution of Cys17, impaired the predominant localization in the filiform apparatus. Cys84 is a conserved cysteine residue that is essential for intramolecular disulfide bridges [57]. Thus, nitrosation could inhibit the secretion and activity of CRP810 attractants immediately after pollen tube arrival at the synergid cell, although the FER-dependency of AtLURE1 inactivation has not been explored.

Altogether, the expression and secretion of a cocktail of CRP810 attractants from synergid cells are tightly controlled via crosstalk between the synergid cells and the egg, central, or ovular sporophytic cells via monitoring of the fertilization success. Future research should examine the *in vivo* regulatory dynamics of the diffusible attractants from synergid cells and how the growth direction of pollen tubes is distantly and temporally controlled toward each ovule to maximize the fertilization success rate in the pistil.

4.3. Reception of LURE peptides by pollen tube receptors

In plants, it has been reported that most secreted peptides/proteins are received by the extracellular domains of receptor-like kinases (RLKs). Among over 600 *RLKs* in *A. thaliana*, more than 450 are predicted to encode single transmembrane proteins with extracellular and cytoplasmic kinase domains [80]. Through AtLURE1-sensitivity screening for pollen-expressed *RLK* mutants, Takeuchi and Higashiyama identified

pollen-specific receptor kinase 6 (PRK6) as an essential receptor for sensing the AtLURE1 attractant peptide [60]. Pollen tubes from PRK6 loss-of-function mutants do not respond to AtLURE1. PRK6 has six leucine-rich repeats (LRRs) and belongs to a subclade consisting of eight PRK family receptors (PRK1–8) in *A. thaliana*. Remarkably, studies of PRK family receptors in tomato and *A. thaliana* have suggested that they regulate pollen tube growth efficiency [37,81]. Consistent with this suggestion, *prk* multiple mutants, such as *prk3 prk6 prk8* and *prk1 prk3 prk6*, showed a dramatic reduction in pollen tube growth and fertility, whereas each single *prk* mutant did not [60]. Additionally, the introduction of the PRK6 deletion mutant into the *prk* mutants implied that the cytoplasmic kinase domain of PRK6 was required for growth regulation when PRK3 was lacking. PRK6 interacts with PRK3 and possibly other PRK receptors, as well as receptor-like cytoplasmic kinases Lost In Pollen tube guidance 1 (LIP1) and LIP2, which have no extracellular domain and are involved in pollen tube growth and AtLURE1 sensing [82]. Despite their relationship with PRKs remaining unknown, another set of pollen-expressed RLKs, MALE DISCOVERER1 (MDIS1), MDIS2, MDIS1-INTERACTING RECEPTOR LIKE KINASE1 (MIK1), and MIK2, is reportedly involved in AtLURE1 sensing [61]. Nevertheless, in combination with these multiple receptor components, PRK6 could play a key role in pollen tube growth and attraction by sensing external ligands, including AtLURE1.

An *in vitro* binding assay demonstrated that AtLURE1 peptides directly interact with the PRK6, and a crystal structure analysis highlighted the complex structure of AtLURE1.2 bound to the PRK6 extracellular domain [57]. Because other LRR-RLKs have binding domains within or between LRR domains for their partner ligands, it is unexpected that AtLURE1.2 binds to the C-terminal loop region of PRK6, which is

between the last LRR and transmembrane domains. Among PRK6 orthologs of *A. lyrata* and another related species, *Capsella rubella*, the C-terminal loop region of PRK6 is less conserved than the LRR domain, despite a set of residues responsible for AtLURE1.2-PRK6 binding, which was confirmed via *in vitro* binding and pollen tube attraction assays, being conserved. This potentially implies that the C-terminal loop region of PRK6 is altered to recognize species-specific attractants. Indeed, AtLURE1.2 exhibited a lower attraction activity toward *A. lyrata* pollen tubes than *A. thaliana* ones [56]. More importantly, *C. rubella* pollen tubes expressing *A. thaliana* PRK6, but not wild-type *C. rubella* pollen tubes, could sense AtLURE1.2 [60], demonstrating that species-specific recognition of the AtLURE1 attractant is mediated through direct binding of PRK6. It is interesting that the second cluster of AtLURE1 peptides (CRP810_3.1/AtLURE1.7 and CRP810_3.2/AtLURE1.8), which are related to but substantially different from AtLURE1.2, also requires PRK6 for pollen tube response [58] and that *C. rubella* appears to have no *Arabidopsis* LURE1 orthologs (Fig. 2). Therefore, it would be interesting to investigate how these variable attractant ligands from each species are recognized by PRK6 or related receptors at the atomic level.

In *A. thaliana*, the AtLURE1 receptor PRK6 is expressed in the mature pollen grain and localized at the tip, even in pollen tubes germinated *in vitro* (a condition without the stigma and style) [60]. However, the *in vitro* pollen tube cannot be adequately attracted to the ovule or purified AtLURE1 peptides [60,69]. Although several chemical compounds derived from female tissues have been reported to stimulate pollen tube germination and growth [83,84], it remains unknown at a molecular level how attractant receptors of the pollen tube are activated after growing through the stigma and style.

In *T. fournieri*, it has been shown that pollen tube competency required for sensing attractants is activated by growth through the stigma/style and receiving sporophytic female factors [51,52]. In a semi-*in vitro* assay, the competency of pollen tube responsiveness to TfLURE2 peptides required a sufficient style length and enough time after emerging from the style [85]. In contrast, irrespective of style length, TfLURE2 binding at the pollen tube tip was detected 12 h, but not 6 h, after pollination, suggesting that the full acquisition of pollen tube competency and TfLURE2 reception are separately regulated during growth processes in *T. fournieri* pollen tubes. After emerging from the style, pollen tube competency is induced by an ovular factor named AMOR [86]. AMOR consists of an arabinogalactan polysaccharide that is generally found in arabinogalactan proteins (AGPs), and likely contains β-(1,3)-galactan main chains with the terminal 4-O-methyl-glucuronosyl galactose. Additionally, the terminal disaccharide structure, the *β* isomer of methyl-glucuronosyl galactose, is required to cause pollen tube competency to respond to ovules as well as purified recombinant TfLURE1 and TfLURE2. To understand the AMOR-controlled intercellular communication required for pollen tube competency, it is necessary to examine whether an endogenous AMOR has a peptide backbone encoded by ovule-expressed genes, the enzyme biosynthesizing the AMOR sugar chain, and how the pollen tube receives the terminal disaccharide structure.

5. Conclusions and perspectives

In summary, substantial progress in research concerning angiosperm male-female communication has been made in the last two decades by identifying key molecules, including CRP ligands and their receptors. LURE-type CRPs, as a cocktail of attractants,

463 are key ligands that ensure efficient and robust pollen tube attraction in a
464 species-specific manner. Although angiosperms have diverse repertoires of
465 synergid-expressed *CRP* genes [45,70], core eudicot species may continue to employ
466 LURE-type CRPs as attractant peptides [51,56]. Nevertheless, LURE-type CRPs exhibit
467 striking sequence divergence among species, unlike other reproductive CRPs, such as
468 LAT52, RALFs, STIG1/GRI, and EC1, orthologs of which can be broadly found via
469 simple homology searches in angiosperm species, including basal angiosperms.
470 Experimental identification of synergid-secreted attractants in many angiosperm species
471 is important for understanding the evolution of pollen tube attractants, which underlies
472 species-specific but common mechanisms in pollen tube guidance. Furthermore, as
473 reproductive CRPs generally undergo rapid molecular evolution with conserved
474 cysteine frameworks, which could allow us to track homologous peptides among related
475 species, it will be interesting to explore the molecular evolution of various types of
476 reproductive CRPs using emerging genome data from angiosperm species as well as
477 other land plants.

478 Understanding the molecular entities and mechanisms of intercellular signaling
479 in plant reproduction could lead to technologies that overcome fertility problems in
480 certain conditions, as well as interspecific reproductive barriers. It could be relatively
481 easy to apply peptides serving as extracellular ligands to manipulate signaling events in
482 reproductive processes. For instance, the introduction of *A. thaliana* LURE1 into *T.*
483 *fournieri* or maize ZmEA1 into *A. thaliana* has been demonstrated to overcome
484 interspecific incompatibility in pollen tube attraction toward the ovule on the medium
485 ([56,63]; Video 1). Because there are multiple barriers in each male-female
486 communication step, the pyramiding of multiple signal manipulations could be useful

for overcoming reproductive barriers *in vivo* and understanding species-specific mechanisms in angiosperm reproduction.

Finally, the tip-growing pollen tube is a great model system for examining the molecular dynamics involved in polarized cell growth, which is mediated via secreted peptide ligands and their receptor complexes. For instance, the AtLURE1-induced asymmetric accumulation of PRK6 at the pollen tube tip was observed before the growth direction was morphologically changed [60]. Furthermore, the pollen tube could have an integration mechanism for multiple ligand-receptor signaling, which includes cell-autonomous controls, such as autocrine RALFs-ANXs/BUPSs signaling, and non-cell-autonomous controls, such as AtLURE1-PRK6 signaling. Further identification of extracellular and intracellular components involved in these signaling pathways is required to improve our understanding. In addition, it is important to identify other peptide-receptor pairs, including experimentally unexplored CRP ligands and pollen tube receptors for *Arabidopsis* CRP810 peptides other than AtLURE1, *Torenia* LUREs, and maize ZmEA1. In the next decade, I expect that angiosperm male-female communication studies will collectively reveal how the pollen tube achieves species-specific rendezvous in the pistil via integrity maintenance and efficient directional growth coordinated by multiple peptide-receptor signaling.

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Figure legends

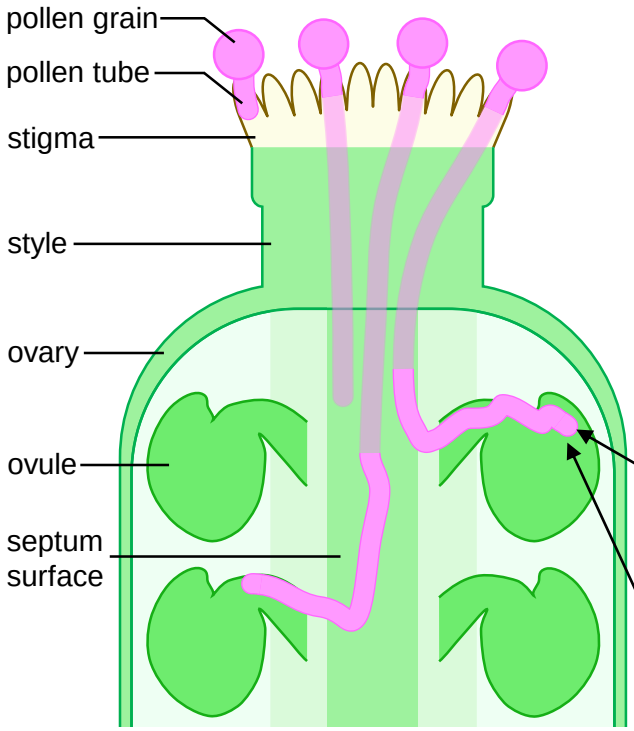
Fig. 1. Schematic illustration of angiosperm sexual reproduction events with reported functional CRPs. Pollen tubes germinated from pollen grains grow into the stigma and enter the ovary through transmitting tissue of the style. After entering on the septum surface, pollen tubes target ovules in a one-to-one manner. The light pink color of pollen tubes indicates a part of pollen tubes growing in the transmitting tissue of the style and ovary. Each CRP has distinct properties, especially the conserved cysteine number (Cys) and partner receptors (shown at the right). Pink and green characters indicate the expression of each factor in male pollen and female tissues, respectively.

Fig. 2. Phylogenetic tree of *CRP810* genes from *A. thaliana* (blue), *A. lyrata* (red), and *C. rubella* (green). Putative coding regions of their genomic sequences of *CRP810* genes, identified by a homology search against each species' nucleotide database on Phytozome (https://phytozome.jgi.doe.gov/), were used. Therefore, this tree contains additional *CRP810* genes from *A. lyrata* and *C. rubella* and presents a slightly different phylogenetic relationship from a previously drawn tree [58], which lacks a certain number of sequences, probably due to incomplete protein annotation. The tree was constructed by a neighbor-joining method with bootstrap values as percentages using MEGA X software [87]. Bootstrap values ≥70 are indicated. The scale indicates the number of substitutions per site.

Fig. 3. Confocal images of ovules expressing CRP810 peptides fused with a yellow fluorescent protein (Citrine), driven by each native promoter. Green and magenta indicate the Citrine fluorescence signal and autofluorescence of ovules, respectively. For

AtLURE1.2, CRP810_3.1/AtLURE1.7, CRP810_3.2/AtLURE1.8, and CRP810_4/XIUQIU1, higher magnification images for synergid cells and micropyle are also shown. Note that, although most of the Citrine-fused CRP810 showed polarized localization around the filiform apparatus, the localization patterns could be slightly different from that of GFP-fused CRP810 in previous reports [56,58,59].

Video 1. Time-lapse imaging of an interspecific pollen tube attraction assay. As previously reported [56], *T. fournieri* ovules expressing AtLURE1.2 were able to attract *A. thaliana* pollen tubes on the medium. The transgenic *T. fournieri* ovule micromanipulated using a glass needle was placed in front of the pollen tube tip and moved once to observe the chasing behavior of the *A. thaliana* pollen tube toward the micropyle of heterogeneous ovules. Two synergid cells of the *T. fournieri* ovule and pollen tubes of *A. thaliana* are labeled with a green fluorescent protein (GFP). An arrowhead indicates the pollen tube tip. Time counter, mm:ss.



pollen germination & self-incompatibility response

SCR/SP11	8 Cys	DEFL	SRK
PrsS	4 Cys	S-protein	PrpS
LAT52	6 Cys	Ole e I	LePRK2

pollen tube growth

RALF4/19	4 Cys	RALF	ANX, BUPS, LRXs
LeSTIG1	16 Cys	STIG1	LePRK2

pollen tube attraction

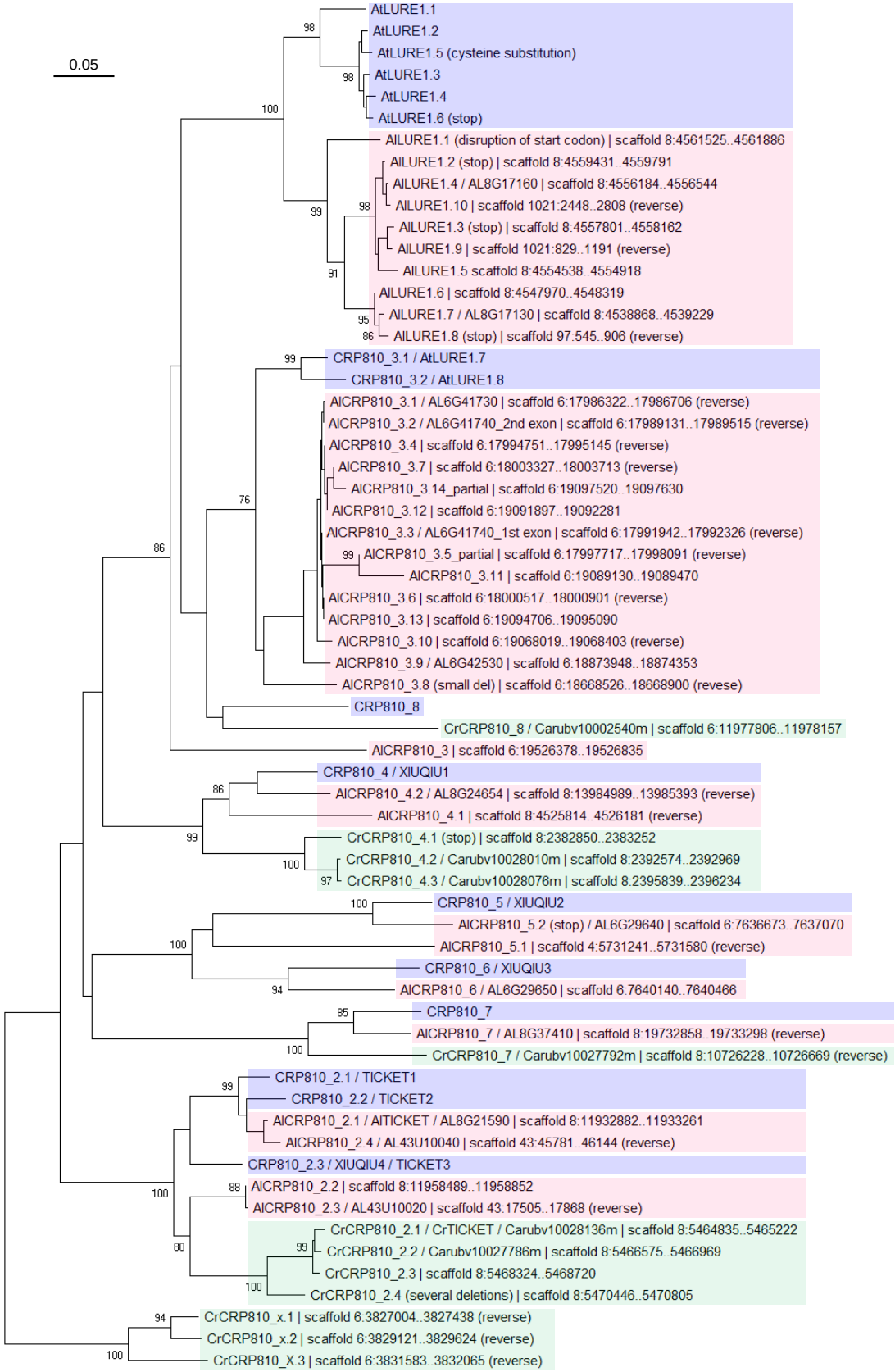
LURE-type CRPs	6 Cys	DEFL	PRK6, MDIS, MIK

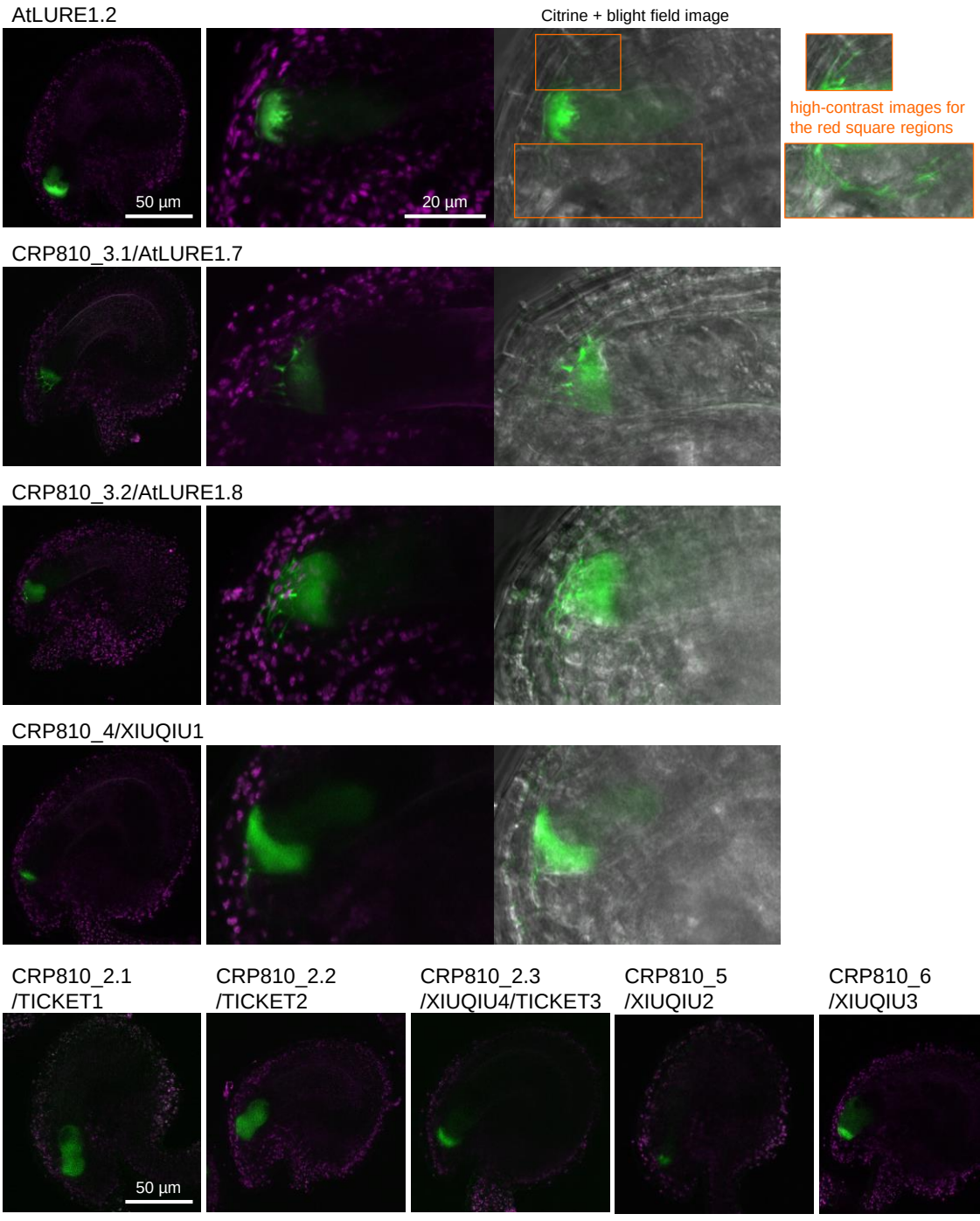
pollen tube rupture

ZmES4	8 Cys	DEFL	KZM1?
RALF34?	4 Cys	RALF	ANX?, BUPS?
CRP2460?	8 cys	Thionin	?

gamete activation & fertilization

EC1	6 Cys	LTP	?


0.05
AtLURE1.1
AtLURE1.2
AtLURE1.5 (cysteine substitution)
AtLURE1.3
AtLURE1.4
AtLURE1.6 (stop)
AlLURE1.1 (disruption of start codon) | scaffold 8:4561525..4561886
AlLURE1.2 (stop) | scaffold 8:4559431..4559791
AlLURE1.4 / AL8G17160 | scaffold 8:4556184..4556544
AlLURE1.10 | scaffold 1021:2448..2808 (reverse)
AlLURE1.3 (stop) | scaffold 8:4557801..4558162
AlLURE1.9 | scaffold 1021:829..1191 (reverse)
AlLURE1.5 scaffold 8:4554538..4554918
AlLURE1.6 | scaffold 8:4547970..4548319
AlLURE1.7 / AL8G17130 | scaffold 8:4538868..4539229
AlLURE1.8 (stop) | scaffold 97:545..906 (reverse)
CRP810_3.1 / AtLURE1.7
CRP810_3.2 / AtLURE1.8
AlCRP810_3.1 / AL6G41730 | scaffold 6:17986322..17986706 (reverse)
AlCRP810_3.2 / AL6G41740_2nd exon | scaffold 6:17989131..17989515 (reverse)
AlCRP810_3.4 | scaffold 6:17994751..17995145 (reverse)
AlCRP810_3.7 | scaffold 6:18003327..18003713 (reverse)
AlCRP810_3.14_partial | scaffold 6:19097520..19097630
AlCRP810_3.12 | scaffold 6:19091897..19092281
AlCRP810_3.3 / AL6G41740_1st exon | scaffold 6:17991942..17992326 (reverse)
AlCRP810_3.5_partial | scaffold 6:17997717..17998091 (reverse)
AlCRP810_3.11 | scaffold 6:19089130..19089470
AlCRP810_3.6 | scaffold 6:18000517..18000901 (reverse)
AlCRP810_3.13 | scaffold 6:19094706..19095090
AlCRP810_3.10 | scaffold 6:19068019..19068403 (reverse)
AlCRP810_3.9 / AL6G42530 | scaffold 6:18873948..18874353
AlCRP810_3.8 (small del) | scaffold 6:18668526..18668900 (revese)
CRP810_8
CrCRP810_8 / Carubv10002540m | scaffold 6:11977806..11978157
AlCRP810_3 | scaffold 6:19526378..19526835
CRP810_4 / XlUQlU1
AlCRP810_4.2 / AL8G24654 | scaffold 8:13984989..13985393 (reverse)
AlCRP810_4.1 | scaffold 8:4525814..4526181 (reverse)
CrCRP810_4.1 (stop) | scaffold 8:2382850..2383252
CrCRP810_4.2 / Carubv10028010m | scaffold 8:2392574..2392969
CrCRP810_4.3 / Carubv10028076m | scaffold 8:2395839..2396234
CRP810_5 / XlUQlU2
AlCRP810_5.2 (stop) / AL6G29640 | scaffold 6:7636673..7637070
AlCRP810_5.1 | scaffold 4:5731241..5731580 (reverse)
CRP810_6 / XlUQlU3
AlCRP810_6 / AL6G29650 | scaffold 6:7640140..7640466
CRP810_7
AlCRP810_7 / AL8G37410 | scaffold 8:19732858..19733298 (reverse)
CrCRP810_7 / Carubv10027792m | scaffold 8:10726228..10726669 (reverse)
CRP810_2.1 / TICKET1
CRP810_2.2 / TICKET2
AlCRP810_2.1 / AlTICKET / AL8G21590 | scaffold 8:11932882..11933261
AlCRP810_2.4 / AL43U10040 | scaffold 43:45781..46144 (reverse)
CRP810_2.3 / XlUQlU4 / TICKET3
AlCRP810_2.2 | scaffold 8:11958489..11958852
AlCRP810_2.3 / AL43U10020 | scaffold 43:17505..17868 (reverse)
CrCRP810_2.1 / CrTICKET / Carubv10028136m | scaffold 8:5464835..5465222
CrCRP810_2.2 / Carubv10027786m | scaffold 8:5466575..5466969
CrCRP810_2.3 | scaffold 8:5468324..5468720
CrCRP810_2.4 (several deletions) | scaffold 8:5470446..5470805
CrCRP810_x.1 | scaffold 6:3827004..3827438 (reverse)
CrCRP810_x.2 | scaffold 6:3829121..3829624 (reverse)
CrCRP810_X.3 | scaffold 6:3831583..3832065 (reverse)


AtLURE1.2
Citrine + blight field image
high-contrast images for the red square regions
50 µm
20 µm
CRP810_3.1/AtLURE1.7
CRP810_3.2/AtLURE1.8
CRP810_4/XIUQIU1
CRP810_2.1 /TICKET1
CRP810_2.2 /TICKET2
CRP810_2.3 /XIUQIU4/TICKET3
CRP810_5 /XIUQIU2
CRP810_6 /XIUQIU3
50 µm