Peptide signalling during the pollen tube journey and double fertilization

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Abstract

Flowering seed plants (angiosperms) have evolved unique ways to protect their gametes from pathogen attack and from drying out. The female gametes (egg and central cell) are deeply embedded in the maternal tissues of the ovule inside the ovary, while the male gametes (sperm cells) are enclosed in the vegetative pollen tube cell. After germination of the pollen tube at the surface of papilla cells of the stigma the two immobile sperm cells are transported deep inside the sporophytic maternal tissues to be released inside the ovule for double fertilization. Angiosperms have evolved a number of hurdles along the pollen tube journey to prevent inbreeding and fertilization by alien sperm cells, and to maximize reproductive success. These pre-zygotic hybridization barriers require intensive communication between the male and female reproductive cells and the necessity to distinguish self from non-self interaction partners. General molecules such as nitric oxide (NO) or gamma-aminobutyric acid (GABA) therefore appear to play only a minor role in these species-specific communication events. The past 20 years have shown that highly polymorphic peptides play a leading role in all communication steps along the pollen tube pathway and fertilization. Here we review our current understanding of the role of peptides during reproduction with a focus on peptide signalling during self-incompatibility, pollen tube growth and guidance as well as sperm reception and gamete activation.

Key words: Cysteine rich peptide (CRP), fertilization, pollen tube, receptor-like kinase (RLK), reproductive isolation, self-incompatibility.

Introduction

Angiosperms are characterized by their unique mode of sexual reproduction including a double fertilization process, in which two sperm cells fuse with the egg and central cell, respectively, to form both embryo and endosperm respectively (Raghavan, 2003). The immobile sperm cells need to be delivered as cargo toward the deeply embedded female gametophyte with the help of a pollen tube, representing the fastest growing plant cell. The entire process consists of a number of successive steps initiated after pollen deposition on the stigma, and its adhesion, hydration and germination to produce the pollen tube. Elongation at the tip is typical for polar pollen tube growth. Pollen tubes first penetrate the intercellular space at the stigma and style, and then pave their way along the nutritious extracellular matrix of transmitting tract tissues towards the ovary. Transmitting tract tissues differ significantly between species and may consist of specialized cell files associated with the vascular tissue in plants like Arabidopsis (Crawford and Yanofsky, 2008) and maize (Lausser et al., 2010) or hollow walls in plants like the lily (Lord, 2003).

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After perceiving signals from the ovule, pollen tubes exit the transmitting tract, grow on the surface of the placenta and migrate up the funiculus towards the micropylar opening of the ovule, where they enter the embryo sac (for review see Higashiyma and Takeuchi, 2015). This pathway is typical for many angiosperms including the model plant *Arabidopsis*. Due to anatomical differences and the presence of a single ovule per ovary, this stage of the pollen tube journey from the transmitting tracts towards the micropyle was named as ovarian cavity growth in the grasses (Lausser and Dresselhaus, 2010) and likely depends on mechanical rather than on chemotactic cues. Finally, after arrival at the egg apparatus (consisting of the egg cell and accessory synergid cells) the pollen tube decelerates its speed, grows around the synergid cells (Leshem et al. 2013) and ultimately bursts into the receptive synergid cell thereby releasing its cargo for double fertilization. Cell fusions (plasmogamy) take place after both sperm cells arrive at the junction between egg and central cell where they are activated (Sprunck et al. 2014). Subsequently the fertilized egg cell (zygote) develops into an embryo and the fertilized central cell forms the endosperm, the two major components of angiosperm seeds (Weterings and Russell, 2004).

In order to achieve and maximize their reproductive success, flowering plants have evolved complicated signalling mechanisms to assure and regulate every step during the pollen tube journey and subsequent double fertilization (Hamamura et al., 2012; Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Bleckmann et al., 2014). Recent studies have revealed that peptide signalling plays a leading role during short-range signalling along the whole pollen tube pathway, the regulated release of its cargo and for gamete activation.

In general, plant peptides can be categorized into two classes: secreted peptides and non-secreted peptides. Secreted peptides can be further divided into two major classes: cysteine-rich peptides (CRPs) and non-CRPs (NCRPs). There are 432 NCRP genes annotated in the *Arabidopsis* genome including post-translationally modified small peptides and some proteins, which are not yet classified (Huang et al., 2015). Post-translationally modified peptides are generated from the cell where signal molecules are released to the distance, which adds to the signal sensitivity (Busch and Benfey, 2010). The affinity of their binding to the cognate receptors is presumably high, as the working concentration is usually in the nM range or even below (Matsubayashi et al., 2001). After receptor activation, peptide-receptor complexes are often internalized to the cytosol through endocytosis, where the receptor undergoes degradation or recycling (Geldner and Robatzek, 2008).

In the various male-female interactions during plant reproduction, small peptides secreted either from pollen grains or tubes (male gametophyte), from embryo sac cells (female gametophyte) or the sporophytic maternal tissues of the pistil play critical roles, for example, in self-incompatibility responses, pollen tube growth support and guidance, pollen tube burst and gamete activation (Fig. 1). The role of peptides in these reproductive processes will be outlined in more detail below. An overview about the signalling peptides discussed below is shown in Table 1.

### Roles of plant peptides in self-incompatibility responses

Recognition of self-pollen, pollen from the same or alien species occurs after pollen grains have been deposited at papilla cells of the stigma. Self-incompatibility (SI) responses are thereafter activated in many plant species to prevent inbreeding by pollen of the same species. This pre-fertilization reproduction barrier promotes outcrossing and thus avoids the negative effects associated with inbreeding depression (Losdat et al., 2010). After secretion they diffuse to neighbouring cells over short distances (a few cell files) to bind cognate receptors, but long-distance signalling from the root to the shoot has also been reported (Tabata et al., 2014). A concentration gradient is generated from the cell where signal molecules are released to the distance, which adds to the signal sensitivity (Busch and Benfey, 2010). The affinity of their binding to the cognate receptors is presumably high, as the working concentration is usually in the nM range or even below (Matsubayashi et al., 2001). After receptor activation, peptide-receptor complexes are often internalized to the cytosol through endocytosis, where the receptor undergoes degradation or recycling (Geldner and Robatzek, 2008).

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In self-incompatibility responses selective inhibition of germination or growth of self-pollen grains (or tubes) is initiated to prevent self-fertilization and thus contributes to maintaining the gene pool and genetic variation in plants (Kitashiba and Nasrallah, 2014).

Self-incompatibility responses are controlled by the multi-allelic S-locus in many plant species (Takayama and Isogai, 2005). The S-locus genes determining self-incompatibility in the Brassicaceae, for example, consist of those encoding the polymorphic and haplotype-specific cysteine rich protein/S-locus protein 11 (SCR/SP11) and the S-locus receptor kinase (SRK) (Stein et al., 1991; Schopfer et al., 1999; Takayama et al., 2000). Stigmas of SRK gain-of-function transformants reject pollen grains expressing SCR/SP11 of the same haplotypes (Takashiki et al., 2000; Silva et al., 2001). Moreover, transfer of the SRK and SCR/SP11 genes from one S-locus of self-incompatible Arabidopsis lyrata to self-fertile A. thaliana is sufficient to cause self-incompatibility in this species (Nasrallah et al., 2002).

SCR/SP11 is a defensin-like CRP with eight conserved cysteine residues, consisting of about 50 amino acids after signal peptide cleavage (Takayama et al., 2000; Table 1). In situ hybridization analyses showed that SCR/SP11 is mainly expressed in the sporophytic tapetum cell layer, and, in some species, in the microspores. During pollen maturation, the peptide is released to the anther locule where it accumulates on the pollen coats. Upon pollination, SCR/SP11 diffuses to the papilla cells, where it binds the receptor SRK at the plasma membrane (Iwano et al., 2003).

SRK is a receptor-like-kinase (RLK) expressed in the stigma papilla cells prior to flower opening (anthesis). It forms a homodimer constitutively, which binds SCR/SP11 with high efficiency (Takayama et al., 2001). Nuclear magnetic resonance (NMR) analysis showed that SCR/SP11 folds into a αβ-sandwich structure containing three anti-parallel β-sheets and one α-helix, which are connected by loop regions. The conserved cysteine residues form four disulfide bonds, which stabilize the peptide structure. The binding-specificity of SCR/SP11 to its receptor SRK is mainly determined by the two loop regions. However, the overall binding capacity additionally depends on the whole conformation of the peptide (Chookajorn et al., 2004). As mentioned above the S-locus genes harbour high polymorphisms as sequence similarity between two individuals (haplotypes) is usually less than

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### Table 1

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Receptors</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCR/SP11</td>
<td>SRK</td>
<td>Self-incompatibility response</td>
</tr>
<tr>
<td>ProS</td>
<td>PrpS</td>
<td></td>
</tr>
<tr>
<td>LatS2</td>
<td>LePRKs</td>
<td>Pollen tube germination and tip growth</td>
</tr>
<tr>
<td>STIG1</td>
<td>LePRKs</td>
<td></td>
</tr>
<tr>
<td>CLE45</td>
<td>SKM1 &amp; SKM2</td>
<td></td>
</tr>
<tr>
<td>SCA1TP5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Plantacyanin</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Chemocyanin</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>ZmEA1</td>
<td>—</td>
<td>Ovular pollen tube guidance</td>
</tr>
<tr>
<td>LUREs</td>
<td>LIP1 &amp; LIP2*</td>
<td>Micropylar pollen tube guidance</td>
</tr>
</tbody>
</table>

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Fig. 1. Schematic representation of secreted peptides (see also Table 1) and their receptors involved in pre-zygotic communication of reproductive cells. (A) Pollen-pistil interactions in self-incompatibility responses, pollen tube growth and guidance. Please note that LIP1 and LIP2 (one asterisk) are not direct receptors, but are proposed to represent part of the LURE receptor complex. (B) Enlarged image of an ovule shortly before sperm cell release is shown [see also highlighted frame in (A)]. The role of peptides and receptors is detailed in the text. The interaction of RALFs (two asterisks) with the FER/SR KZM1** and ANX1 & ANX2** is illustrated. (C) Schematic representation of secreted peptides (see also Table 1) and their receptors involved in pre-zygotic communication of reproductive cells. (A) Pollen-pistil interactions in self-incompatibility responses, pollen tube growth and guidance. Please note that LIP1 and LIP2 (one asterisk) are not direct receptors, but are proposed to represent part of the LURE receptor complex. (B) Enlarged image of an ovule shortly before sperm cell release is shown [see also highlighted frame in (A)]. The role of peptides and receptors is detailed in the text. The interaction of RALFs (two asterisks) with the FER/SR KZM1** and ANX1 & ANX2** is illustrated.
50% (Schopfer et al., 1999). Variation is especially high in the loop 1 region of SCR/SP11, the hypervariable (HV) domain, which further supports the hypothesis that these sites serve as decisive regions of a certain haplotype (Mishima et al., 2003).

The S-allele-specific interaction between SCR/SP11 and SRK triggers SI responses leading to pollen rejection. SRK undergoes auto-phosphorylation after ligand-receptor binding (Cabrillac et al., 2001; Takayama et al., 2001). Then the activated kinase domain recruits and works together with an M-locus protein kinase (MLPK), a receptor-like cytoplasmic kinase, to phosphorylate Armadillo Repeat-Containing protein 1 (ARC1) (Murase et al., 2004; Kakita et al., 2007). ARC1 acts as an E3 ligase and mediates the ubiquitination and subsequent degradation of stigma membrane protein Exo70A1, which disturbs pollen grain hydration, and ultimately blocks successful pollen tube germination (Stone et al., 2003; Samuel et al., 2009).

The SI response may also occur later during pollen tube growth inside the transmitting tract. While the above type of SI was named as sporophytic SI (SSI), the later type is considered as gametophytic SI (GSI) as it is mainly controlled by the S-alleles of the vegetative pollen tube cell. GSI involving peptide-mediated communication is best understood in poppy (Papaver rhoes) (Eaves et al., 2014). Here the S-determinant (PrsS) encodes a CRP secreted from pistil cells (Foote et al., 1994), which interacts with the pollen tube S-determinant PrpS and triggers a Ca²⁺-dependent signalling network in incompatible pollen tubes. This in turn results in inhibition of pollen tube growth and ultimately leads to programmed cell death (PCD). PrpS is a small transmembrane protein located at the pollen tube plasma membrane and is involved in Ca²⁺ influx (Wheeler et al., 2009). In addition to Ca²⁺ and K⁺ influx, acidification of the cytosol has been reported recently (Wilkins et al., 2015). Altogether PrsS interaction with its receptor PrpS triggers a number of downstream events such as depolymerization of F-actin, phosphorylation and thereby inhibition of sPPases (soluble inorganic pyrophosphatases), activation of p56 MAPK (mitogen-activated protein kinase), an increase in ROS (reactive oxygen species) and NO as well as the activation of caspase-3-like/DEVDase enzymes, which finally leads to PCD (for review see Eaves et al., 2014).

### Table 1. Peptide ligands involved in signalling processes during pollen germination, tube growth and double fertilization

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide family</th>
<th>Length (aa)*</th>
<th>Genus</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAT52</td>
<td>KT/0le 1** (CRP)</td>
<td>144</td>
<td>Tomato</td>
<td>Pollen hydration and PT*** growth</td>
<td>Muschietti et al., 1994</td>
</tr>
<tr>
<td>STIG1</td>
<td>STIG1 (CRP)</td>
<td>120</td>
<td>Tomato</td>
<td>Promotes PT growth</td>
<td>Tang et al., 2004; Huang et al., 2014</td>
</tr>
<tr>
<td>STIG1</td>
<td>STIG1 (CRP)</td>
<td>123</td>
<td>Tobacco/Petunia</td>
<td>Secretion of pistil exudates</td>
<td>Goldman et al., 1994; Verhoeven et al., 2005</td>
</tr>
<tr>
<td>SCR/SP11</td>
<td>DEFL (CRP)</td>
<td>50–77</td>
<td>Brassica</td>
<td>Pollen SI determinant Stylar PT adhesion</td>
<td>Schopfer et al., 1999</td>
</tr>
<tr>
<td>SCA</td>
<td>LTP (CRP)</td>
<td>91</td>
<td>Lily</td>
<td>PT guidance?</td>
<td>Park et al., 2000; Mollet et al., 2000</td>
</tr>
<tr>
<td>LTP5</td>
<td>LTP (CRP)</td>
<td>93</td>
<td>Arabidopsis</td>
<td>PT growth</td>
<td>Chae et al., 2010; Chae and Lord, 2011</td>
</tr>
<tr>
<td>PrsS</td>
<td>S1</td>
<td>120</td>
<td>Poppy</td>
<td>Sporophytic SI determinant</td>
<td>Foote et al., 1994</td>
</tr>
<tr>
<td>Chemocyanin</td>
<td>Phytocyanin</td>
<td>96</td>
<td>Lily</td>
<td>PT reorientation</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td>Plantacyanin</td>
<td>Phytocyanin</td>
<td>96</td>
<td>Arabidopsis</td>
<td>PT guidance?</td>
<td>Dong et al., 2005</td>
</tr>
<tr>
<td>CLE45</td>
<td>CLV3/ESR</td>
<td>12</td>
<td>Arabidopsis</td>
<td>Promotes PT growth at high temperature</td>
<td>Endo et al., 2013</td>
</tr>
<tr>
<td>EA1</td>
<td>EA1-like</td>
<td>49</td>
<td>Maize</td>
<td>PT guidance and growth arrest</td>
<td>Márton et al., 2005; Márton et al., 2012</td>
</tr>
<tr>
<td>LUREs</td>
<td>DEFL (CRP)</td>
<td>62–70</td>
<td>Torenia</td>
<td>PT attraction</td>
<td>Okuda et al., 2009; Kanaoka et al., 2011</td>
</tr>
<tr>
<td>LUREs</td>
<td>DEFL (CRP)</td>
<td>71–75</td>
<td>Arabidopsis</td>
<td>PT attraction</td>
<td>Takeuchi and Higashiyama, 2012</td>
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<tr>
<td>RALFs</td>
<td>RALF (CRP)</td>
<td>−50</td>
<td>Arabidopsis</td>
<td>H⁺/Ca²⁺ homeostasis</td>
<td>Haruta et al., 2014***</td>
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<td>ES1-4</td>
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<td>61</td>
<td>Maize</td>
<td>PT burst</td>
<td>Amien et al., 2010; Woriedh et al., 2015</td>
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<tr>
<td>PMEI1</td>
<td>PMEI (CRP)</td>
<td>−150–160</td>
<td>Maize</td>
<td>PT burst</td>
<td>Woriedh et al., 2013</td>
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<tr>
<td>EC1</td>
<td>ECA1 (CRP)</td>
<td>101–131</td>
<td>Arabidopsis</td>
<td>Sperm cell activation</td>
<td>Sprunck et al., 2012; Sprunck et al., 2014</td>
</tr>
</tbody>
</table>

* Length of the predicted mature peptides in amino acids (aa); ** KT/0le 1, homology to Kunitz-type trypsin inhibitors/Ole e 1 pollen allergens; *** PT, pollen tube; **** RALFs are strongly expressed in pollen tubes and bind to the FER/STN1 receptor. A signalling role during reproduction remains to be demonstrated.
Roles of peptides for pollen tube growth behaviour

During compatible interactions the vegetative cell of compatible pollen grains produces a pollen tube. Signals from both the pistil and the pollen maintain the polarized growth pattern of pollen tubes by controlling the cytoskeleton dynamics and vesicle trafficking (Chebl et al., 2013; Guan et al., 2013). In tomato (Solanum lycopersicum), a member of the Kunitz trypsin inhibitor-like CRPs, LAT52, participates in pollen tube germination. Pollen grains injected with antisense LAT52 germinate abnormally (Twell et al., 1989; Muschietti et al., 1994). LAT52 binds specifically to two pollen receptor kinases LePRK1 and LePRK2. The 122 amino acids at the C-terminus of LAT52 are responsible for this receptor-binding activity. Both LAT52 and LePRKs are expressed in mature pollen grains, while only the expression of LePRKs increases significantly after germination, consistent with the finding that the interaction of their encoded proteins is undetectable when pollen tubes elongate further. There exist two different forms of LAT52 before and after germination. It is possible that pollen monitors its germination status by LAT52, since the binding affinity of LAT52 to LePRKs changes over time, and the smaller form of LAT52 expressed after germination is unable to bind LePRKs efficiently (Tang et al., 2002). During pollen germination LePRK1 and LePRK2 are detected in a ~400 kDa protein complex, which is disrupted in vitro after germination initiation in the presence of a 3–10 kDa fraction of style extract. It was therefore hypothesized that a smaller extracellular peptide may induce complex dissociation after germination (Wengier et al., 2003). LeSTIG1 (see below) appears to represent such a candidate peptide.

After germination, pollen tubes penetrate through the style and transmitting tract and grow towards the ovary. Pollen tubes grow considerably more slowly in culture medium compared with in vivo growth, indicating that factors derived from the pistil promote their growth. One example is the tomato small CRP peptide LeSTIG1, which is secreted from stigma tissues. Treatment with recombinant LeSTIG1 promotes pollen tube elongation (Tang et al., 2004). LeSTIG1 encodes a 143-amino-acid peptide containing 16 conserved cysteine residues at its C-terminus. LeSTIG1 is expressed during pistil maturation, and its expression is restricted to the stigma and upper section of the style in the mature pistil. After being processed, a 7 kDa peptide is released and accumulated at the pollen tube surface where it interacts with LePRK receptors. The tetrapeptide FNYF in the conserved C-terminal region of LeSTIG1 is the core domain sufficient for binding to LePRKs. The three pollen tube receptors LePRK1-3 share similar expression patterns in the flower, but they possess different binding affinities with different peptides (Kim et al., 2002). For example during pollen germination LePRK2 binds LAT52, while during pollen tube elongation LePRK1 and LePRK2 bind STIG1. By interacting with different peptides, LePRKs regulate pollen tube germination and growth at different stages (Tang et al., 2002). The cytoplasmic kinase domain of LePRKs is able to interact with KPP, a plant-specific Rop GTPase exchange factor (GEF) (Kaothien et al., 2005). It was further reported that ROS inhibits pollen tube growth in vitro, therefore it is possible that LePRKs regulate pollen tube growth through induction of downstream signalling events including the regulation of ROS production (Zhang et al., 2008). Recently LeSTIG1 was reported to be able to bind phospholipids like P(I)3P. Thus it may indirectly regulate the cell redox state (Huang et al., 2014). LeSTIG1 homologues in other species appear to have other functions, for example, STIG1 regulates the release timing of the secretion of exudates from pistils in petunia (Petunia hybrida) and tobacco (Nicotiana tabacum) (Verhoeven et al., 2005).

Pollen tube growth is also known to be sensitive to fluctuating environmental conditions such as temperature changes. A post-translationally modified NCRP peptide CLV3/ESR-related 45 (CLE45) was recently reported to maintain seed production under high temperature conditions (Endo et al., 2013). CLE45 is expressed in the stigma and vascular tissues. Upon temperature shift from 22°C to 30°C its distribution expands to the transmitting tract, where it binds two RLKs (i.e. SKM1 and SKM2) at the surface of pollen tubes. Synthetic mature CLE45 peptide therefore promotes pollen tube growth in vitro at 30°C but not at 22°C. This signal thus prolongs the growing period of a pollen tube instead of affecting elongation rate in vivo, probably through maintaining mitochondrial activity at higher temperature, which eventually contributes to adequate fertilization (Endo et al., 2013).

Inside the transmitting tract, pollen tube growth is guided and supported by many chemical cues. Several members of the LTP subfamily of CRPs, such as SCA, are expressed in the pistil to support pollen tube growth (Cha and Lord, 2011). SCA, a 9–10 kDa peptide with a globular structure is stabilized by four conserved disulfide bonds and secreted from epidermal cells into the extracellular matrix (ECM) of the transmitting tract cells. The peptide is believed to participate in pollen tube adhesion-mediated guidance because it is positively charged, which facilitates binding to negatively-charged pectin and thus likely results in the adherence of pollen tubes to the transmitting tract surface for growth support (Lord, 2000). SCA is also reported to bind the tip of growing pollen tubes in vitro, and appears in endocytic compartments of pollen tubes in vivo (Kim et al., 2006). It is very likely that these events are caused after ligand-receptor binding, suggesting that SCA conveys a signal to the pollen tube tip (Kim et al., 2006). However, until now receptors and downstream signalling pathway(s) have not yet been described. In Arabidopsis, an SCA-like molecule, LTP5, also possesses a SCA-similar function (Cha et al., 2010).

Additionally, gradients of peptides like plantacyanins have been described to act as guidance signals for polarized tip growth of pollen tubes (Dong et al., 2005). Plantacyanin is a 10 kDa-sized small secreted protein related to a class of basic copper-containing blue proteins (Dong et al., 2005). Plantacyanin is expressed in many tissues. During reproduction mature plantacyanin peptide forms a steep concentration gradient from the stigma towards the ovule, with a lower concentration at the stigma end. This distribution pattern is probably regulated
by a miRNA pathway, which is important for its function (Maunoury and Vaucheret, 2011). If this spatial pattern is disturbed by plantacyanin overexpression, pollen tubes expand randomly at stigmatic papilla cells showing defective polar growth. Due to its copper-binding motif, it is predicted to display a high redox potential with a copper molecule exposed on the surface. This feature reveals a role of plantacyanin in ROS metabolism and thereby affecting pollen tube growth (Dong et al., 2005). Chemocyanin in lily (Lilium longiflorum) is a homologue of plantacyanin, acting similarly to chemotropic molecules for pollen tube growth. However, chemocyanin lacks copper-binding capability due to a single amino acid substitution at the binding site, and its specific mechanism regulating pollen tube remains unclear (Kim et al., 2003).

Roles of peptides for ovular pollen tube guidance

Based on few eudicotyledonous model species, pollen tube guidance towards and inside ovules was previously divided into two steps: funicular guidance and micropylar guidance (Higashiyama et al., 2003). Due to recent findings the first step, which is mediated by sporophytic signals, was further distinguished into two steps namely pre-ovular and ovular guidance (Higashiyama and Takeuchi, 2015). Micropylar guidance is mediated by gametophytic signals from the embryo sac (Márton and Dresselhaus, 2010). In order to additionally consider the morphology and knowledge from plant families such as the grasses we suggest distinguishing between ovular guidance (mediated by signals derived from sporophytic ovule tissues) and micropylar guidance (guidance mediated by signals from female gametophytic cells). Until now, little is known about the molecular mechanism of sporophytic ovular guidance. Recently, two mitogen-activated protein kinases, MPK3 and MPK6, have been discovered to be involved in ovular guidance (Guan et al., 2014). In vivo pollination assays showed that mpk3 mpk6 pollen tubes were defective in ovule guidance, but micropylar guidance in a semi in vivo pollination assay appeared normal (Guan et al., 2014).

In recent years, elegant studies have elucidated many more details of the molecular mechanism of micropylar guidance (Fig. 2A). In 2001, it was demonstrated using a laser ablation assay that a diffusible attraction signal is derived from the two synergid cells located as part of the egg apparatus close to the egg cell. Using Torenia fournieri as a model—a unique plant with a naked embryo sac that protrudes from the microplye of the ovule—it was shown that the distance of pollen tube attraction by the synergid cell is about 100 μm at most in vitro, indicating that synergid cells produce short-range attractants (Higashiyama et al., 2001). This implied for the first time that attractants are more likely larger molecules such as small secreted peptides rather than small molecules like NO or ROS (Higashiyama et al., 1998; Palanivelu and Preuss, 2006).

The first reported pollen tube attractant was ZmEA1 (Zea mays EGG APPARATUS1), a peptide exclusively expressed in the maize egg apparatus before fertilization. Down-regulation of ZmEA1 expression resulted in misguidance of pollen tubes in the micropylar region of the ovule causing fertilization failure. Therefore, ZmEA1 was considered as a signalling molecule for short-range pollen tube guidance in maize (Márton et al., 2005). ZmEA1 encodes a 94 amino acid hydrophobic precursor protein with a predicted N-terminal transmembrane domain. Mature ZmEA1 was predicted to be N-terminally cleaved to generate a 49 amino acid oligopeptide, which is secreted from the egg apparatus and accumulates in the cell walls of micropylar nucellar cells. If expressed from Arabidopsis synergid cells, ZmEA1 is capable of guiding maize pollen tubes in vitro within a distance of 150 μm towards the micropylar opening of Arabidopsis ovules (Márton et al., 2012). Recent studies further showed that the ZmEA1 peptide interacts with the maize pollen tube tip in a species-specific manner in vitro. After binding, the peptide is immediately internalized into vesicles and degraded (Uebl er et al., 2013).

Fig. 2. Hypothetical model of peptide signalling at the end of the pollen tube journey. (A) Pollen tubes are attracted by LURE and EA1 peptides (blue dots), respectively, released by constitutive secretion (CS) from synergid cells. Unknown receptors (white) interact with cytoplasmic LIP1/2 (violet) associated with the LURE receptor complex. Peptides secreted from the pollen tube may include RALF peptides (brown dots), which interact with the FER/SPN receptor (green) and induce Ca2+ spiking. This activates calcium induced secretion (CIS) of stored secretory vesicles containing ES1-4 (orange dots) and PMEI peptides (grey dots), respectively. Their release leads to osmotic burst of the pollen tube tip after inactivation of PME (dark green) and ion influx by channel (pink) opening. (B) Pollen tube burst induces a Ca2+ elevation in the egg cell. Whether CIS leads to release of the sperm activator peptide EC1 (yellow dots) remains to be determined. The EC1 receptor at the sperm surface is yet unknown.
ZmEA1 belongs to the EA1-like (EAL) family peptides, a novel class of hydrophobic and polymorphic peptides, which do not exist in eudicots (see below). Until now only one additional member of this family, EAL1 (EA1-like1), has been functionally characterized in maize and was shown to be involved in regulating cell fate determination of female gametophyte cells (Krohn et al., 2012). Homologous sequences are also present in other grasses such as Brachypodium distachyon (purple false brome), Sorghum bicolor (sorghum) and Oryza sativa (rice), but not in A. thaliana or other dicotyledonous plant species (Dresselhaus et al., 2011), suggesting that EA1-like peptides are probably Gramineae-specific.

In dicotyledonous plants, another group of peptides was identified as pollen tube attractants that act in a species-preferential manner. CRPs of the DEFL subclass named as LUREs (LURE1 and LURE2) were first identified as attractant molecules in Torenia fournieri involved in micropylar pollen tube guidance. Predicted mature LUREs in Torenia species consist of 62–70 amino acid residues (Table 1) and contain six cysteine residues. Down-regulation of LURE genes by injection of morpholino antisense oligomers into the embryo sac cells of T. fournieri strongly decreases the attraction frequency of pollen tubes by the ovule (Okuda et al., 2009). Recombinant LURE1 and LURE2 proteins expressed in Escherichia coli exhibit similar pollen tube attraction activity in vitro. 40 pM of LUREs (~1000 molecules) are sufficient to attract pollen tubes. LUREs belong to the most strongly expressed genes in synergid cells. The encoded peptides are secreted towards the filiform apparatus and likely generate the necessary concentration gradient around the micropyle, which is important for pollen tube attraction and guidance towards the egg apparatus (Okuda et al., 2009). Related TcCRP1 from T. concolor, which differs from LURE1 of T. fournieri by eight amino acids, also showed species-preferential activity (Kanaoka et al., 2011) indicating that this step of the pollen tube journey is strongly regulated and represents a major pre-zygotic hybridization crossing barrier in plants. Further support for this hypothesis comes from research in A. thaliana, where five CRPs (AtLURE1.1–AtLURE1.5; predicted mature peptides are 71–75 amino acids in length), were identified as attractants controlling pollen tube guidance into the micropyle of Arabidopsis ovules. AtLUREs are also secreted from the synergid cells and spread around the funiculus. With the exception of AtLURE1.5, which lacks one conserved cysteine, all AtLUREs are capable of attracting pollen tubes (Takeuchi and Higashiyama, 2012). Moreover, it was shown that AtLUREs attracted pollen tubes of A. thaliana better than tubes of A. lyrata indicating that they act in a species-preferential manner. The comparison of LUREs from Torenia and Arabidopsis species shows little homology (Higashiyama and Takeuchi, 2015), but based on the number and arrangement of cysteine residues, they are all grouped into the defensin-like (DEFL) subclass of CRPs. In flowering plants DEFL genes form the largest CRP subfamily, with 317 DEFL genes in Arabidopsis. They partly appear to have evolved by tandem and segmental duplication events (Takeuchi and Higashiyama, 2012). We assume that this type of CRP peptide is also used by other eudicotyledonous plant species to attract pollen tubes into the ovule. However, the low sequence conservation between Torenia and Arabidopsis LUREs indicates that it will be difficult to predict these from the large number of DEFL genes in other species. Experimental pollen tube guidance assays have to be applied to test candidates identified by bioinformatic approaches.

Receptors perceiving LURE signals are not yet identified in any plant species. However, two Arabidopsis receptor-like cytoplasmic kinases (RLCKs), called Lost In Pollen Tube Guidance 1 (LIP1) and 2 (LIP2), were recently identified to be involved in AtLURE1-mediated micropylar pollen tube guidance (Liu et al., 2013). LIP1 and LIP2 are predominately expressed in pollen tubes and anchored to the inner plasma membrane in the pollen tube tip region via palmitoylation. This localization is essential for their function in pollen tube guidance control. In lip1 lip2 double mutants the attraction of pollen tubes towards AtLURE1 was significantly reduced. The observation that loss of LIP1/2 function resulted in more than 60% reduction of male transmission in vivo while retaining 70% of the in vitro response activity towards AtLURE1 suggests that there might be other RLCKs participating in AtLURE1-mediated pollen tube guidance signalling, or that LIP1 and LIP2 are likely to simultaneously participate in other peptide-mediated signalling pathways during pollen tube guidance (Liu et al., 2013). Because LIP1 and LIP2 lack extracellular domains to bind AtLUREs directly, it is more likely that they function as components of a receptor complex (Fig. 2A). A receptor complex often consists of two RLKs, a receptor containing an extracellular domain for ligand interaction and a co-receptor RLK with a shorter extracellular domain, and a RLCK lacking extracellular domains. Two examples are the BR11-BAK1-BSK1 receptor complex in BR signalling and FLS2-BAK1-BIK1 receptor complex in innate immunity signalling (Kim and Wang, 2010; Lu et al., 2010). The identification of LIP1 and LIP2 will now facilitate identification and characterization of the other components of the receptor complex in pollen tube guidance in Arabidopsis.

Finally, it is important to note that loss of function of MYB98, a transcription factor specifically expressed in synergid cells of Arabidopsis, resulted in more severe defects in pollen tube guidance compared to AtLURE1 RNAi lines (Takeuchi and Higashiyama, 2012), indicating that there might be additional pollen tube attractants expressed in synergid cells in eudicots, which need to be identified in the future. In contrast it was shown that maize ovules lacking embryo sacs showed the same pollen tube attraction phenotype as ZmEA1-RNAi lines (Lauser et al., 2010) suggesting that the conserved ZmEA1 peptide likely represents the sole micropylar attractant in the grasses.

Peptides involved in pollen tube reception
After reaching the micropyle, growth of the pollen tube ceases, and the pollen tube enters the female gametophyte where it grows beyond the filiform apparatus to enter the synergid cell at a more distant site, where it ruptures to release its contents, including the two sperm cells (Lesheen et al., 2013).
This process is called pollen tube reception (Kasahara et al., 2005).

One RLK, FERONIA/SIRENE (FER/SRN), which localizes predominately at the surface of synergid cells, plays an essential role in growth arrest of the pollen tube (Escobar-Restrepo et al., 2007). The extracellular domain of FER/SRN is variable in different plant species, consistent with species-specificity observed in this pollen tube reception event. It was previously suggested that FER/SRN may interact with an unidentified ligand on the surface of the pollen tubes or with a signalling ligand derived from the pollen tube (Rotman et al., 2003; Huck et al., 2003). Due to recent findings (see below) we speculate that CRPs of the RALF family may represent these ligands (Fig. 2A). While FER/SRN is localized to the female gametophyte and lacks in pollen tubes, ANXUR1 (ANX1) and ANXUR2 (ANX2), two close homologues of FER/SRN in the same RLK subfamily, are expressed in pollen and were reported to be involved in maintaining pollen tube integrity during growth. Simultaneous inactivation of ANX1 and ANX2 results in growth arrest and burst of pollen tubes in vitro (Boisson-Dernier et al., 2009; Miyazaki et al., 2009). These findings further indicate that the release of sperm cells is controlled by signalling events occurring between both female and male gametophytes. Therefore the identification of the ligands of ANX1 and ANX2 as well as FER/SRN is another goal to be accomplished in the near future. Recently a small and secreted peptide of the RALF (Rapid Alkalization Factor) subclass of CRPs was reported to bind FER/SRN in roots and induce the suppression of cell elongation in the primary root. RALF-FER/SRN interaction causes inhibition of the plasma membrane H+-ATPase thereby mediating a pH change in the cytoplasm (Haruta et al., 2014). In addition, RALF induces the increase of Ca²⁺ concentration in the cytosol (Pearce et al., 2001; Haruta and Constabel, 2003; Haruta et al., 2008), which may also affect pollen tube growth and burst. Whether RALFs are also capable of interacting with ANX1 and ANX2 remains to be shown. In maize RALF encoding genes belong to the strongest expressed genes in pollen tubes (M. Woriedh and T. Dresselhaus, unpublished) pointing to the possibility of both ANX1 and ANX2 as well as FER/SRN interaction with RALF peptides during reproduction.

_Zea mays Embryo Sac1–4_ were the first peptide genes showing a specific expression pattern in reproductive cells. They encode members of the DEFL subclass of CRPs and were shown to be specifically expressed in the whole female gametophyte with strongest expression in the synergid cells (Cordts et al., 2001). Later it was demonstrated that they induce pollen tube burst in a species-specific manner (Amien et al., 2010). Application of ZmES4 led to quick depolarization of the pollen tube plasma membrane, induced potassium channel KZM1 opening and thus K⁺ flux. Whether the activity of other channels is changed due to membrane depolarization is unknown. However, K⁺ flux and influx of other ions such as Na⁺ and Ca²⁺ and sugars likely leads to water uptake, osmotic changes and cytoskeleton disassembly. This ultimately results in pollen tube rupture at the very tip, the weakest point of the pollen tube due to the lack of callose-containing cell wall material at this region. Recently, it was reported that another small apoplastic protein of the PMEI (pectin methyl esterase inhibitor) subgroup of CRPs is strongly expressed in both gametophytes of maize. ZmPMEI1 inhibits PMEs (pectin methyl esterase) whose activity is required to remove methyl and demethoxy groups from homogalactururonan (a major class of pectin and thus of the pollen tube cell wall) and thereby disturbs the integrity and stiffness of the elongating pollen tube apex. Application of recombinant ZmPMEI1 to growing pollen tubes leads to burst at the subapical region. The current model is that the CRPs ZmES1–4 and ZmPMEI1 act in concert to induce pollen tube rupture upon arrival of the pollen tube at the synergid cell (Woriedh et al., 2013; see also Fig. 2A). Whether secretion of these peptides/small proteins is induced by the FER/SRN signalling pathway has to be shown in further experimentation.

### Peptides involved in gamete activation

After release, one sperm cell fuses with the egg cell to form the embryo and the other one fuses with the central cell to initiate the development of the triploid endosperm. This process, double fertilization, represents a (the) major characteristic of flowering plants and is at the same time the last possibility to control compatibility of gametes and thus may represent a final pre-zygotic hybridization barrier. Many signalling events are involved, including cell recognition, gamete activation, plasma membrane adhesion and fusion as well as nuclear fusion (karyogamy). Until now, only one peptide class has been identified playing an essential signalling role in this process (Fig. 2B). Five _Arabidopsis_ peptides, named Egg Cell1 (EC1), are secreted by the egg cell after sperm cell release (Sprunk et al., 2012). EC1s belong to the EC1 subgroup of ECA1 (Early Culture Abundant 1) gametogenesis-related CRPs (Sprunk et al., 2014) and consist of ~100–130 amino acids after signal peptide removal. They contain six conserved cysteine residues and two conserved signature sequence motifs (Sprunk et al., 2012). All five _Arabidopsis_ **EC1** genes (**EC1.1**–**EC1.5**) function redundantly to activate sperm cells, thereby enabling them to fuse with the two female gametes. Simultaneous inactivation of all five **EC1** genes leads to inhibition of gamete fusion and reduced seed set. Multiple pollen tubes are attracted in the quintuple mutant and to some extent sperm cells acquire late competence for fusion 2–3 days after pollination (Rademacher and Sprunk, 2013).

Upon sperm cell arrival, EC1s are released from the egg cell and trigger the re-localization of HAP2/GCS1 (Sprunk et al., 2012), a sperm-specific integral membrane protein, which is required for gamete fusion. From _hap2/gcs1_ mutant pollen tubes sperm cells are properly released, but fail to fuse with female gametes (Mori et al., 2006; von Besser et al., 2006). Exogenous application of EC1 triggers HAP2/GCS1 redistribution from the endomembrane system to the sperm.
cell surface and thus activates sperm cells to fuse with the two female gametes (Sprunck et al., 2012).

EC1 proteins are found in all angiosperm species investigated, including the basal angiosperm Amborella trichopoda (Sprunck et al., 2012). It will now be interesting to find out whether Arabidopsis EC1 is capable of activating sperm cells of other plant species and vice versa. Moreover, the central cell also contains CRPs (Schmid et al., 2012) and it will now be interesting to find out to what extent it contributes to sperm cell activation and the precise signalling during double fertilization. First studies point in this direction; in the female gametophyte mutant glc (glauce) one sperm cell successfully fuses with the egg cell, while the second sperm cell fails to fuse with the central cell. glc mutants lack a central cell-expressed BAHD acyltransferase, which is involved in secondary metabolism and possibly required to generate a central cell-derived signalling molecule (Leshem et al., 2012).

Conclusions

In the past few years, an increasing knowledge of peptide signalling has contributed significantly to the understanding of the molecular mechanisms regulating plant reproduction, especially along the pollen tube journey and during double fertilization. A number of secreted peptides mainly of various CRP subclasses were demonstrated to participate in pollen grain recognition at the stigma, in pollen tube growth support and guidance, in attracting the tube to enter the embryo sac and in mediating gamete interaction (Figs 1, 2).

Peptides involved in double fertilization appear to belong to large gene families with multiple members. Most members show similar expression patterns and function redundantly, as revealed by the studies of LUREs (Takeuchi and Higashiyama, 2012), ES1–4 (Amien et al., 2010; Woriedh et al. 2015) and EC1s (Sprunck et al., 2012). CRP peptide subfamilies like LTPs and RALFs are strongly expressed along the pollen tube journey, but precise roles are not yet known. Other peptide families appear to play a predominant role after fertilization such as the MEG1 CRPs and AE peptides, whose diverse roles are highlighted elsewhere in this special issue (Gutierrez-Marcos and Ingram, 2015). However, considering that 756 CRP and 432 non-CRP genes were annotated in the Arabidopsis genome, of which the majority is expressed specifically during reproduction and seed development (Huang et al., 2015), it can be expected that many more functions about peptide signalling during these important biological processes will be elucidated in the near future.

As ligands, apoplastic peptides are supposed to be perceived by one or more cell surface receptors. However for the majority of peptides functioning or being expressed along the pollen tube pathway and in double fertilization, the corresponding receptors have not been identified. RLKs, with more than 630 members, comprise the largest receptor family in plants (Shiu and Bleecker, 2001), and most peptide receptors identified so far belong especially to the Leucine-Rich-Repeat (LRR)-type RLKs (Shiu and Bleecker, 2003). Many RLKs are expressed in plant gametophytes (Wang et al., 2014; Rutley and Twell, 2015; see also cited references in these articles) implying that they may play important roles in reproduction, probably by acting as peptide receptors during male-female communication. Until now few peptide ligand-receptor pairs involved in plant reproduction have been identified (Fig. 1). Novel and high-throughput biochemical methods (Uebler and Dresselhaus, 2014) combined with highly sensitive mass spectrometric instruments will now help to identify more receptors of peptide ligands in the near future.

A further task for the future will be to elucidate how peptide signals are transduced into cellular responses. Many well-studied peptides are reported to bind extracellular domains of receptor oligomers, for example SCR binds an SRK homodimer (Takayama et al., 2001), which causes autophosphorylation of the RLK kinase domain and subsequently activates phosphorelay cascades (Guo et al., 2013). Downstream responses then include fast responses including protein modification (Takayama et al., 2001), protein degradation (Stone et al., 2003), Ca²⁺ fluctuation (Haruta et al., 2008), changes of redox state (Zhang et al., 2008), alteration of cytoskeleton dynamics (Guan et al., 2013) etc. or delayed responses associated with gene expression changes, which have been well studied, for example, during stem cell homeostasis in plants (Richards et al., 2015), but not during reproduction.

In conclusion, our current knowledge about the roles of peptides during pollen tube germination and growth as well as during double fertilization is still very limited. In addition to the induction of signalling pathways they probably have roles such as binding lipids, regulating vesicular trafficking, cell wall integrity and cytoskeleton dynamics, as is the case, for example, for STIG1, which appears to possess multiple roles (see above). How signalling peptides themselves are regulated, both transcriptionally and post-translationally, to generate or maintain specific spatiotemporal expression patterns, also needs to be investigated in more detail. Moreover, exploring the function of the numerous uncharacterized peptides expressed during reproduction (Huang et al., 2015) represents a major task for the plant research community to obtain a more comprehensive understanding of the processes that lead to successful double fertilization or species isolation in plants.

Integrated methods from various fields including mass spectrophotometric imaging and structure biology as well as more advanced live cell and single molecule imaging technologies are now necessary and will allow the study of the dynamics of peptide-ligand interactions and the activated signalling pathway(s) in more detail. Moreover, at present our understanding of peptide signalling during reproduction has been derived from the analysis of few peptides in many plant taxa such as Arabidopsis, Brassica, lily, maize, Petunia, poppy, tobacco, tomato and Torenia (Table 1). It is yet unclear whether the mechanisms described above are conserved among plant species or whether plants species/genera have developed independent peptide signalling mechanisms during reproduction. However, the understanding of peptide based male-female communication likely represents a key to elucidate speciation mechanisms in plants. The various hybridization barriers along the pollen tube journey require
precise and compatible peptide-receptor interactions. Thus this knowledge has great potential for future exploitation in plant breeding to overcome crossing barriers between plant species or even genera that cannot be crossed today. We look forward to more exciting studies about peptide signalling during plant reproduction in the near future.

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