In search of ligands and receptors of the pollen tube: the missing link in pollen tube perception

Said Hafidh*, David Potěšil†‡, Jan Fíla*, Jana Feciková*, Věra Čapková*, Zbyněk Zdráhal†‡ and David Honys*[1]

*Laboratory of Pollen Biology, Institute of Experimental Botany ASCR, Rozvojová 263, 165 02 Praha 6, Czech Republic

†CEITEC – Central European Institute of Technology, Masaryk University, Brno, Czech Republic

‡National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, Czech Republic

Abstract

The journey undertaken by the pollen tube in angiosperms to reach the deeply embedded female gametophyte for fertilization involves persistent guidance by the female gametophyte and accurate perception of the signals by the pollen tube. Several ovule-secreted peptides have been identified. Nevertheless, there are no exact findings on how these signals are perceived by the pollen tube. As a novel approach, we have improvised a modified SIV (semi-*in vivo*) technique, SIV-PS (SIV pollen tube secretome), to perform gel-free LC–MS/MS for high-throughput analysis of pollen-tube-secreted proteins. Our approach has led to the identification of over 1400 protein groups. Among them are pollen-tube-secreted ligands and receptor proteins representing potential male components in perceiving ovule-emitted cues for guidance. The present article reviews the missing link in pollen tube perception and showcases the improvised SIV-PS as a tool for high-throughput and targeted study of the pollen tube secretome.

Introduction

In the 2011 review of Kessler and Grossniklaus [1] entitled 'She's the boss: signaling in pollen tube reception', the authors' choice of title reflected the influence of increasing knowledge on female guidance cues in the last decade and a significant gap in the male counterpart, giving an impression that 'she rules'. With the increasing discoveries of essential male counterparts for signal perception, this view is vindicated, although incomplete.

A number of ovule-secreted peptides have been reported to guide pollen tube entry into the micropyle ([2–8], and for a comprehensive review, see [9]). Several other proteins that are not secreted, but with a similar function, have also been identified. Together, they represent the components of a female gametophyte required for pollen tube guidance. *FER* (*FERONIA*) encodes a receptor-like kinase belonging to the CrRLK1L-1 (*Catharanthus roseus* receptor-like kinase 1-like 1) subfamily of kinases. It contains an N-terminal signal peptide, a transmembrane domain and a putative extracellular carbohydrate-binding malectin-like domain [4,10]. Upon pollen tube arrival, FER is mobilized to the plasma membrane of the filliform apparatus and induces pollen tube burst and sperm cell discharge [4]. *fer* ovules are non-receptive, resulting in pollen tube coiling and block of pollen tube burst [4]. Downstream of FER, is NTA (NORTIA), a MLO (mildew-resistance locus O)-like protein. NTA also contains a signal peptide, seven predicted transmembrane domains and a C-terminal calmodulin-binding domain. During pollen tube reception, NTA shows FER-dependent relocalization to the micropylar end of the synergid cells [11]. NTA also exhibits phenotypic defects similar to *fer*, but at lower frequency [11]. In maize, knockdown of a small defensin-like CRP (cysteine-rich protein), ZmES4 (*Zea mays* embryo sac 4), also mimics the *fer*-like phenotype, and synthetic ZmES4 ligands can induce pollen tube burst *in vitro* [12]. ZmES4 is proposed to impose pollen tube burst through KZM1, a pollen tube *shaker* K^+ channel, and that premature pollen tube rupture is blocked by the activity of two pollen-specific paralogue proteins of FER, ANX (ANXUR) 1 and 2 [12]. Thus FER could sense pollen tube arrival and signal secretion of ZmES4 ligands from the secretory vesicles to trigger pollen tube rupture and release of the twin sperm cells for fertilization [11]. LRE (LORELEI) is a small putative GPI (glycosylphosphatidylinositol)-anchored protein secreted by the filliform apparatus and localized on the plasma membrane [3]. The *lre* phenotype resembles that of *fer*; however, its mechanisms of action is still unknown. LRE could act as a cell-surface receptor of pollen-tube-secreted ligands or as a signalling molecule upon release from the anchor. LUREs are another class of synergid cell small secreted proteins that belong to the defensin-like subgroup of CRPs and are required for pollen tube micropylar guidance [5,6]. In maize, ZmEA1 (*Zea mays* egg apparatus 1) is expressed in synergid cells as well as egg cells and, once secreted, it localizes to the surface of micropylar nucellus cones at the micropylar end of the female gametophyte [2]. Both *in vitro* and SIV (semi-*in vivo*) assays demonstrated that

Key words: cell–cell signalling, chemotaxis, palmitoylation, pollen tube guidance, secretion, semi-*in vivo* pollen tube secretome (SIV-PS).

Abbreviations: ANX, ANXUR; CRP, cysteine-rich protein; CrRLK1L-1, *Catharanthus roseus* receptor-like kinase 1-like 1; ER, endoplasmic reticulum; EXPO, exocyst-positive organelle; FER, FERONIA; GPI, glycosylphosphatidylinositol; LAT52, late anther tomato gene 52; LIP, LOSS IN POLLEN GUIDANCE; LRE, LORELEI; LSP, leaderless secreted protein; NTA, NORTIA; RLCK, receptor-like cytoplasmic kinase; SIV, semi-*in vivo*; SIV-PS, SIV pollen tube secretome; SIV-PT, SIV pollen tube; TGN, *trans*-Golgi network; ZmEA1, *Zea mays* egg apparatus 1; ZmES4, *Zea mays* embryo sac 4.

[1]To whom correspondence should be addressed (email david@ueb.cas.cz).

ZmEA1 possesses species-specific pollen tube attraction activity [13].

In the present mini-review, a few identified key players of the male components in pollen tube perception are discussed. However, the bulk of the present review aims to introduce a modified SIV approach, SIV-PS (SIV pollen tube secretome), as a dedicated tool to facilitate and speed up the discovery of pollen-tube-secreted peptides and metabolites as male gametophyte components during pollen tube signal perception and fertilization.

The missing link in pollen tube perception

Until recently, the only model that exemplified ligand–receptor interactions in pollen tube perception was the interaction of pollen-specific proteins LePRK2 [*Solanum lycopersicum* (formerly *Lycopersicon esculentum*) pollen receptor kinase 2] receptor kinase with LAT52 (late anther tomato gene 52), in what is proposed to be an autocrine signalling system [14,15]. LAT52 was also later shown to mildly interact with PRK4 (pollen receptor kinase 4) to promote pollen hydration and pollen tube growth [16]. Ever since, progress has been made with the recent discovery of LIP (LOSS IN POLLEN GUIDANCE) 1/2, two palmitoylated RLCKs (receptor-like cytoplasmic kinases), which are simultaneously required to perceive signal of recombinant AtLURE1.2 (*Arabidopsis thaliana* LURE 1.2) *in vitro* [17]. Loss-of-function mutation in *lip1*;*lip2* double mutants results in loss of pollen tube guidance and aberrant pectin deposition in pollen tube tips [17]. Other male components with currently unknown mechanisms of action include: AtLTP5 (*Arabidopsis thaliana* lipid-transfer protein 5) [18], a homologue of lily SCA (stigma/stylar cysteine-rich adhesin), pollen-specific receptor kinases ANX1 and ANX2 [19,20] and COBRA-like 10 [21]. ANX1 and ANX2 are male paralogues of female FER/SIRENE. They are among CrRLK1L-1 receptor-like kinases and also contain a putative extracellular carbohydrate-binding malectin-like domain [10]. Both components, *fer* and *anx1*/*anx2*, regulate pollen tube discharge upon arrival at the egg apparatus [4]. The handful of male components discovered so far show defects in pollen tube guidance; however, the mechanism of their signal perception with female-secreted ligands and receptors is still being sought.

Until recently, there has not been a high-throughput approach available to study molecular signals involved in male–female interactions. Progress in this field for the last decade has led to the identification of several female signalling components essential for pollen tube guidance [9]. Pollen tubes embedded deeply in female sporophytic tissues have been a contributing factor to this lack of progress.

However, two consecutive studies explored SIV transcriptomics to identify a subset of genes induced in pollen tubes following pollen interaction with pistils [22,23]. These genes, including three MYB transcription factors MYB97, MYB101 and MYB120, were proposed to be critical for pollen tube growth through the pistil. The authors' follow-up studies made another breakthrough discovery by isolating downstream targets of MYB triple mutants *myb97*;*myb101*;*myb120*, in which pollen tubes with combined mutations failed to enter the micropyle and burst, but instead coiled around the ovules [23]. Some 45 genes were identified to be down-regulated and three up-regulated after pistils were pollinated with *myb97*;*myb101*;*myb120* pollen grains [23]. Most abundant targets included transmembrane transporters (seven of 48), carbohydrate-active enzymes (six of 48) and small secreted proteins (17 of 48). These downstream targets, as well as upstream activators of the three MYB genes, are the male components of pollen tube perception and are most likely to be involved in signal transduction with ovule-secreted attractants.

The task of understanding pollen tube guidance has almost without exception relied on understanding gene-expression patterns followed by reverse genetic analysis. Isolated mutants have been informative, but limited. Global investigation of genes with such function has been impeded by the fact that pollen tubes grow through a mash of female sporophytic tissues which make it nearly impossible to isolate pollen tubes without cross-contamination.

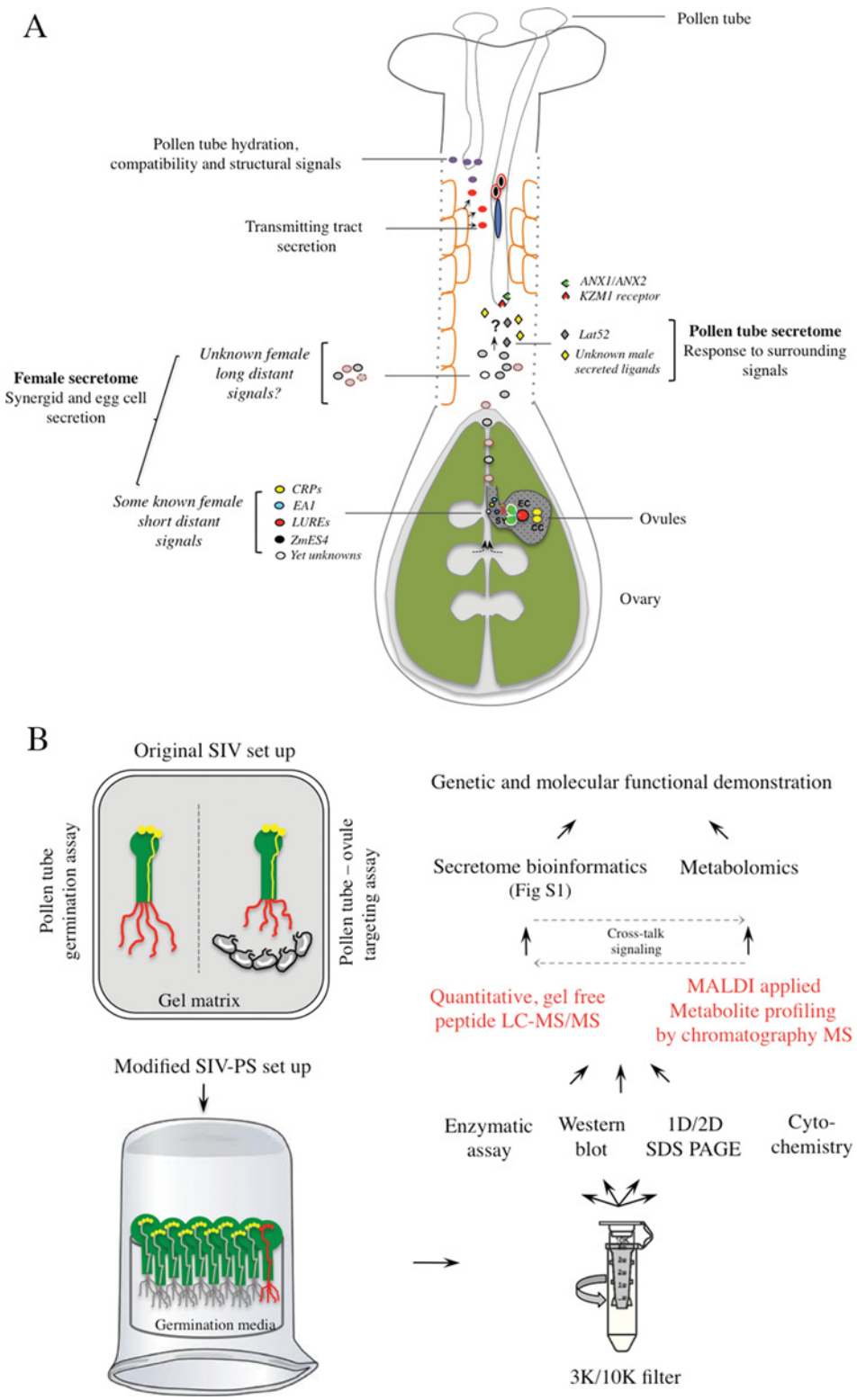
New high-throughput technique for capturing pollen-tube-secreted proteins

In recent work, we have adapted and modified the SIV technique to a set-up that is compatible with downstream 'gel-free' LC–MS/MS analysis, SIV-PS. The method is explained in the legend of Figure 1 showing the schematic arrangement of the set-up. Our approach facilitates high-throughput analysis of pollen-tube-secreted proteins and peptides. This approach is also suitable for performing metabolite profiling using HPLC–MS to identify potential chemosignals including hormones (e.g. abscisic acid), specific amino acids (e.g. γ-aminobutyric acid) and other metabolites that could function in concert with secreted peptides. Few chemoattractants have been reported to have a function in pollen tube guidance [9]. We have captured numerous pollen-tube-secreted proteins including potential pollen tube ligands and receptor proteins with diverse roles in small GTPase signalling, defence and cell wall modification as well as metabolic processes (S. Hafidh, D. Potěšil, J. Fíla, J. Feciková, V. Čapková, Z. Zdráhal and D. Honys, unpublished work). In the present article, we describe this set-up and discuss how to capture pollen-tube-secreted proteins for identification using the SIV-PS '-omic' approach or to use this set-up for more targeted studies of isolated candidate genes.

It best suits to conduct the LC–MS/MS analysis in a quantitative manner. Such an approach will generate peptides with quantitative information which is crucial in determining a bias in protein secretion in a case where a protein secretion is bidirectional originating from both gametophytes or secreted by the transmitting tract tissues of the pistils. Protein accessions observed in control samples, 'pistil-excretome' from excised unpollinated pistils were identified and are subtracted from the list of pollinated pistil

Figure 1 | **Improvised SIV method for capturing and identification of pollen-tube-secreted peptides**

(**A**) Sketch of pollen tube growth through the pistil exemplifying secreted peptides known to be involved in pollen tube perception, with the majority being those identified from the female reproductive tissues (see [9] for a recent review). (**B**) Stepwise guidance on capturing and identification of pollen-tube-secreted proteins and metabolites. On day 1, flowers are emasculated a day before anthesis and netted with nylon mesh to prevent cross-pollination. On day 2, succulent pistils of the emasculated flowers are hand-pollinated. On day 3 (or after a desired length of time, in our case 24 h), pollinated and unpollinated control flowers are collected and pistils are excised approximately 20 mm below stigma shoulders with



a sharp 21 gauge (~3.8 cm) needle. Excised pistils are then placed in a germination chamber filled with pollen tube germination medium SMM-MES [175 mM sucrose, 1.6 mM boric acid (H_3BO_3), 3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.8 mM $MgSO_4 \cdot H_2O$, 1 mM KNO_3 and 25 mM Mes, buffered to pH 5.9] and incubated for another 24 h at 28°C in a humid container. On day 4, the germination media are collected into sterile Falcon tubes and secreted proteins are concentrated using Millipore Amicon Ultra-2 Pre-Launch 3K/10K (3 kDa/10 kDa) filters. Aliquots of protein samples are used to test cytosolic enzyme activity or Western blotting using a cytoplasmic marker to assess levels of cytosolic contamination. Secreted proteins captured can be assessed by 1D/2D SDS/PAGE or by gel-free Orbitrap LC–MS/MS or equivalent. For metabolomics, collected media can be used for HPLC–MS. Several bioinformatics tools are then applied for data analysis. For guidance on bioinformatics tools, see Supplementary Figure S1 at http://www.biochemsoctrans.org/bst/042/bst0420388add.htm.

accessions before proceeding with further analysis. However, this approach is not an exclusive rule of thumb as some accessions observed in the control samples could be truly secreted by the pollen tube, and thus quantitative peptide information is useful to establish the bias and determine the predominant source of secretion. Such conflict arises because of limited peptide counts and close homology between protein accessions. The list of peptides obtained are subjected further to detailed bioinformatics analysis to annotate and establish true secreted proteins. For further guidance on bioinformatics tools and steps to follow, see the Supplementary Figure S1 at http://www.biochemsoctrans.org/bst/042/bst0420388add.htm).

Additionally, this set-up can also be arranged (after optimization) to study the influence of ovule-secreted attractants on pollen tube secretion by incubating pollinated pistils with freshly isolated ovules. A subtractive approach after LC–MS/MS analysis should then be used to exclude accessions identified in unpollinated cut pistils, unpollinated cut pistils incubated with ovules and accessions from incubation of ovules alone. Remaining accessions (not exclusively) can then be considered as a pollen tube secretome stimulated by ovule-secreted attractants and can be compared with a pollen tube secretome in the absence of ovules. A genetic approach and production of synthetic peptides can be employed to validate the function of identified secreted proteins. Moreover, ovule-secreted peptides, metabolites and other chemotactants can also be explored in search of ovule-secreted attractant molecules.

To extend the secretome study, pollen tubes that emerged from the pistil cut-ends can be excised, stored and used for transcriptome studies by NGS (next-generation sequencing), small RNA profiling and total pollen tube proteome analysis, as well as microscopy.

Furthermore, this improvised set-up can be implemented to study mechanisms of secretion under the influence of pharmacological compounds such as brefeldin A, wortmannin and other analogues, or to dissect gene function involved in the secretory pathways.

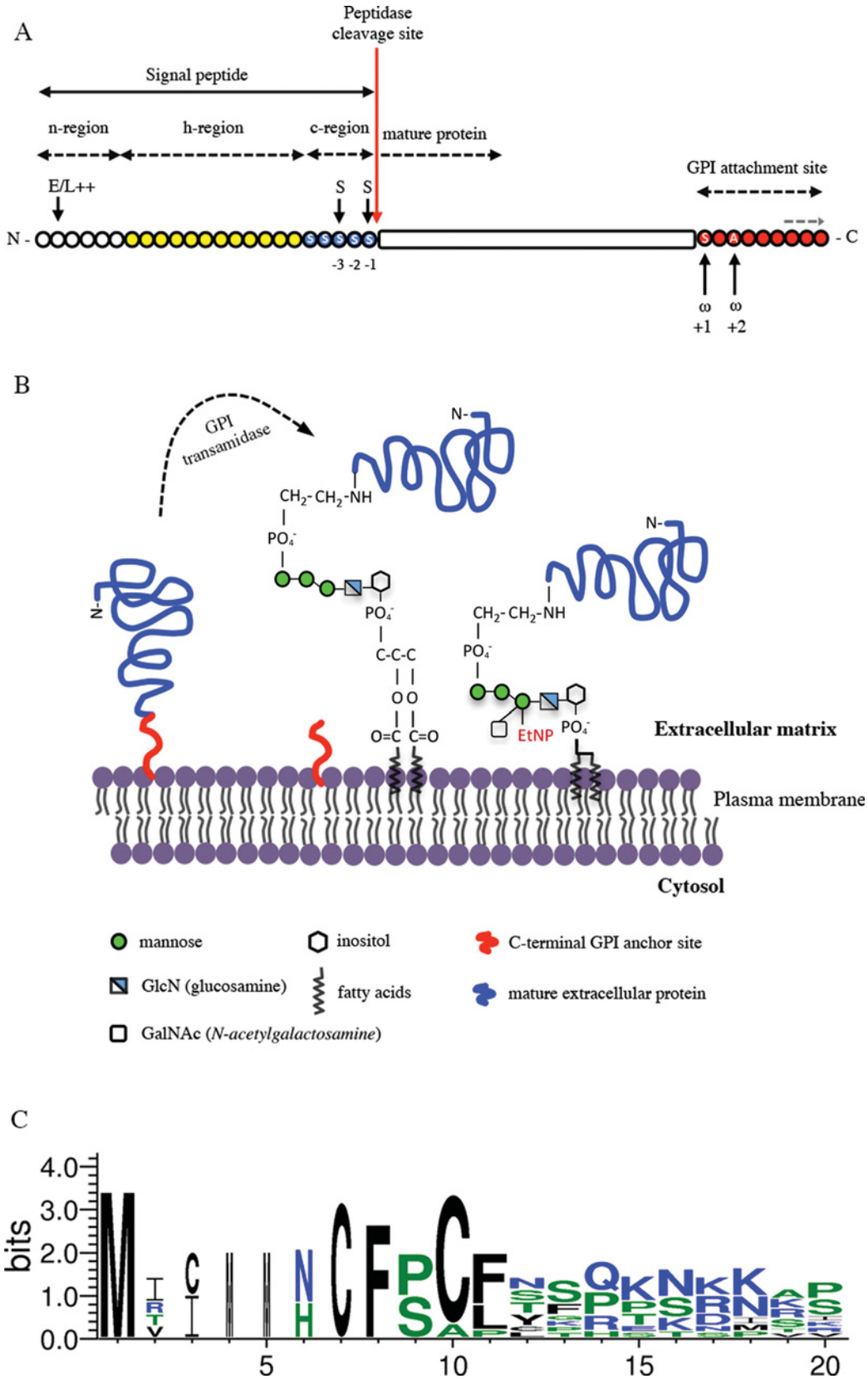
It has yet to be demonstrated how far ovule-secreted attractants, including chemoattractants, can travel up the pistil to guide the pollen tube and how these perceived signals influence pollen tube secretion. Thus, noteworthily, sampling secreted peptides or other attractant molecules is likely to be influenced by the time of pollen tube germination or ovule incubation, and one should expect a differential profile in response to time of incubation corresponding to the stage of pollen tube guidance and receptiveness.

Alternative mechanisms for ligand and receptor secretion by the pollen tube

Conventional or classical protein secretion in plants and in animals is mediated by the ER (endoplasmic reticulum)–TGN (*trans*-Golgi network) system whereby proteins with canonical N-terminal signal peptide are packed into secretory vesicles for secretion to the plasma membrane or extracellular matrix. Once at the ER, the signal peptide is cleaved off and matured proteins are packed for secretion (Figure 2A). Nonetheless, accumulating evidence from plant secretome studies have reported a much larger fractions of unconventionally or non-classically secreted proteins that lack N-terminal signal peptide, termed LSPs (leaderless secreted proteins) [24–27]. In our SIV-PS study, we have identified approximately 20% as classically secreted proteins and a much higher proportion as non-classically secreted proteins by the pollen tube (S. Hafidh, D. Potěšil, J. Fíla, J. Feciková, V. Čapková, Z. Zdráhal and D. Honys, unpublished work). Using combined bioinformatic approaches, we could predict >50% of the identified non-classical proteins as secreted. Current models proposed for the unconventional protein secretion include (i) non-vesicle-mediated secretion possibly by plasma membrane transporters, and/or (ii) vesicle-mediated secretion. With the vesicle secretion mechanisms, three endomembrane compartments have been reported in plants to contribute to LSPs secretion: (i) fusion of the EXPO (exocyst-positive organelle) with plasma membrane, (ii) vacuole–plasma membrane fusion, and (iii) exosome–plasma membrane fusion (reviewed in [24,26,27]). Two additional vesicular secretion mechanisms of LSPs have been described in animals and yeast: (iv) fusion of lysosomes with the plasma membrane, and (v) reverse pinocytosis (microvesicle shedding/exovesicles/apoptotic blebs) [24]. Nevertheless, existence of EXPOs in plants is still debatable [28]. Thus, when studying pollen-tube-secreted proteins, non-classically secreted proteins also require careful analysis for their potential role in pollen tube growth as well as signal perception.

In a search for receptors, conventional receptor proteins involved in signal transduction [4,10] are known to possess transmembrane domains with either single or multiple α-helices spanning the plasma membrane once or multiple

Figure 2 | Anchored proteins as potential secreted receptors of the pollen tube



(**A**) An example (*Arabidopsis* LRE) of a secreted GPI-anchored protein with a potential role as synergid cell-surface receptor during pollen tube signal perception. Glypiated proteins constitute a signal peptide at the N-terminus for entry into the ER–TGN secretory pathway, and a GPI-anchor attachment site at the C-terminus for membrane attachment. There are three elements of the signal peptide motif: n-region, polarity region; h-region, hydrophobic region; c-region, cleavage site for protein maturation. Pollen-tube-secreted GPI-anchored proteins could perceive signal secreted by the female gametophyte. The h-region was determined using the method of Kyte and Doolittle [32], the n-region was predicted using the Zimmerman method [33], the ω-GPI attachment site was determined using big-PI (http://mendel.imp.ac.at/gpi/plant_server.html) [34]. (**B**) Anchoring of matured GPI-anchor protein to plasma membrane once at the extracellular matrix is facilitated through attachment of glycolipid at the C-terminus via an ethanolamine phosphate (EtNP) bridge and two fatty acids of the phosphatidylinositol group. Anchored proteins can be released by the activity of phospholipase C, making GPI-anchored proteins as dynamic receptors. (**C**) Conserved potential palmitoylation region among the RLCKs of the VII subfamily enriched in SIV-PTs.

times (such as G-protein-coupled receptors) to attach the polypeptide chains into the lipid bilayer. Generally, secreted proteins are not known to contain transmembrane domains, particularly at the N-terminus end of the protein. Therefore, when analysing the pollen tube secretome for receptors, it is worth also investigating protein post-translational modifications that could anchor secreted protein to the plasma membrane. GPI-anchoring is a post-translational modification of a protein by the attachment of a GPI molecule (glycolipid) at the ω-site C-terminal end to facilitate protein association with the plasma membrane once secreted to the extracellular matrix (Figure 2B). Anchored proteins can serve as cell-surface receptors to transduce signals from bound ligand molecules. GPI-anchored proteins are known to be essential in germline development [29]. In *Arabidopsis*, LRE represents a classically secreted protein with a signal peptide at the N-terminus and a GPI-anchor-attachment site at the C-terminus (Figures 2A and 2B). The significance of the LRE GPI-anchor site is yet to be demonstrated, but it is undoubtedly critical for LRE attachment to the plasma membrane of the synergid cells and therefore for LRE function. If attached, LRE could potentially function as synergid cell-surface receptor to perceive pollen-tube-secreted ligands and guide the pollen tube through the micropylar entry. Discovery of an LRE-binding partner will clarify the LRE's mode of action. COBRA-like 10 is another GPI-anchored protein that mediates female-guided directional pollen tube growth [21]. It localizes at the apical plasma membrane of the pollen tube and requires proper GPI processing and its hydrophobic residues at the C-terminus for its function [21]. Moreover, mutations of several components of the initial GPI-anchor processing complex, GPI–GnTs (N-acetylglucosaminyltransferases), such as SETH1, SETH2 and PNT1 (PEANUT1), all causes defects in pollen germination and tube growth [30,31]. The *Arabidopsis* genome encodes over 200 GPI-anchored proteins with many showing pollen-specific or pollen-enriched profiles [30]. Thus GPI-anchored proteins could play a substantial part in pollen tube signal perception by bridging the two gametophytes during signal transduction. For specific studies of GPI-anchored proteins, an SIV-GPI-anchored protein set-up can be arranged whereby, before collection of the secretome, pollen tubes can be treated with phospholipase C to release the anchored proteins to the free medium.

Other potential pollen tube receptors are proteins undergoing post-translational palmitoylation. Palmitoylation is the covalent attachment of a fatty acid, such as the C_{16} palmitic acid, to specific cysteine residues of a protein. Palmitoylation enhances the surface hydrophobicity and membrane affinity of the modified protein. Palmitoylated proteins are not necessarily secreted and thus they might not be part of the secretome. Nevertheless, their essential role as receptors was demonstrated by the impairment of pollen tube guidance of *lip1* and *lip2* mutants [17]. Both LIP1 and LIP2 proteins contain N-terminal palmitoylation sites that mediate their membrane localization in pollen tubes and are essential for their proper function [17]. Furthermore, our analysis for the palmitoylation sites of the nine members of the RLCK VII subfamily (including LIP1 and LIP2) that were enriched in SIV-PT transcriptome [17] revealed highly conserved cysteine residues at the N-terminus of all members, implying canonical palmitoylation modification of these proteins (Figure 2C). All group members also lack N-terminal signal peptide and are neither predicted to be secreted by SecretomeP bioinformatics tool (results not shown). Therefore, apart from transmembrane receptors, other post-translationally modified proteins could play the signal-perception role during fertilization and are therefore worth investigating.

Future perspectives

The SIV technique has proved very useful in understanding pollen tube perception and guidance by the female gametophyte at the transcriptome level [22,23]. Likewise, modified SIV-PS approaches for gel- and label-free LC–MS/MS analyses as well as metabolite profiling offer a powerful method to investigate several aspects of male–female interaction. Candidates identified by SIV-PS can be tested directly by subcellular protein targeting and synthetic polypeptide assay on SIV-grown pollen tubes. Employing genetics to investigate the role undertaken by candidate proteins can adequately extend this analysis. Unlike genetic approaches, secretome studies provide a more comprehensive picture at an instant. Conversely, this approach is limited to predominantly secreted proteins and requires a careful set-up with numerous controls to eliminate unfavourable secretion and cytoplasmic contamination. Furthermore, this set-up can be used to perform metabolite profiling and to investigate

secretion of chemoattractants that has long been speculated in long-distance guidance of the pollen tube. Undoubtedly, ongoing efforts, including emerging mathematical modelling and computational approaches, will provide better insights into establishing the ligands and receptors involved in male–female gamete interaction for successful sexual reproduction.

Acknowledgements

We gratefully acknowledge Mark Johnson, Alexander Leydon and Christine Beale of Brown University for training on the SIV technique during the RCN (Research Coordination Network) pollen workshop.

Funding

This work was supported by the Czech Science Foundation [grant numbers P501/11/P321 and P501/11/1462], the Czech Ministry of Education, Youth and Sport [project number LD13049] and by the 'CEITEC – Central European Institute of Technology' project [grant number CZ.1.05/1.1.00/02.0068] from the European Regional Development Fund.

References

1 Kessler, S.A. and Grossniklaus, U. (2011) She's the boss: signaling in pollen tube reception. Curr. Opin. Plant Biol. **14**, 622–627
2 Márton, M.L., Cordts, S., Broadhvest, J. and Dresselhaus, T. (2005) Micropylar pollen tube guidance by egg apparatus 1 of maize. Science **307**, 573–576
3 Capron, A., Gourgues, M., Neiva, L.S., Faure, J.E., Berger, F., Pagnussat, G., Krishnan, A., Alvarez-Mejia, C., Vielle-Calzada, J.P., Lee, Y.R. et al. (2008) Maternal control of male-gamete delivery in *Arabidopsis* involves a putative GPI-anchored protein encoded by the *LORELEI* gene. Plant Cell **20**, 3038–3049
4 Escobar-Restrepo, J.M., Huck, N., Kessler, S., Gagliardini, V., Gheyselinck, J., Yang, W.C. and Grossniklaus, U. (2007) The FERONIA receptor-like kinase mediates male–female interactions during pollen tube reception. Science **317**, 656–660
5 Okuda, S., Tsutsui, H., Shiina, K., Sprunck, S., Takeuchi, H., Yui, R., Kasahara, R.D., Hamamura, Y., Mizukami, A., Susaki, D. et al. (2009) Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature **458**, 357–361
6 Takeuchi, H. and Higashiyama, T. (2012) A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*. PLoS Biol **10**, e1001449
7 Kanaoka, M.M., Kawano, N., Matsubara, Y., Susaki, D., Okuda, S., Sasaki, N. and Higashiyama, T. (2011) Identification and characterization of TcCRP1, a pollen tube attractant from *Torenia concolor*. Ann. Bot. **108**, 739–747
8 Takeuchi, H. and Higashiyama, T. (2012) A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*. PLoS Biol. **10**, e1001449
9 Dresselhaus, T. and Franklin-Tong, N. (2013) Male–female crosstalk during pollen germination, tube growth and guidance, and double fertilization. Mol. Plant **6**, 1018–1036
10 Boisson-Dernier, A., Kessler, S.A. and Grossniklaus, U. (2011) The walls have ears: the role of plant CrRLK1Ls in sensing and transducing extracellular signals. J. Exp. Bot. **62**, 1581–1591
11 Kessler, S.A., Shimosato-Asano, H., Keinath, N.F., Wuest, S.E., Ingram, G., Panstruga, R. and Grossniklaus, U. (2010) Conserved molecular components for pollen tube reception and fungal invasion. Science **330**, 968–971
12 Amien, S., Kliwer, I., Márton, M.L., Debener, T., Geiger, D., Becker, D. and Dresselhaus, T. (2010) Defensin-like ZmES4 mediates pollen tube burst in maize via opening of the potassium channel KZM1. PLoS Biol. **8**, e1000388
13 Márton, M.L., Fastner, A., Uebler, S. and Dresselhaus, T. (2012) Overcoming hybridization barriers by the secretion of the maize pollen tube attractant ZmEA1 from *Arabidopsis* ovules. Curr. Biol. **22**, 1194–1198
14 Tang, W., Ezcurra, I., Muschietti, J. and McCormick, S. (2002) A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2. Plant Cell **14**, 2277–2287
15 Johnson, M.A. and Preuss, D. (2003) On your mark, get set, GROW! LePRK2–LAT52 interactions regulate pollen tube growth. Trends Plant Sci. **8**, 97–99
16 Chang, F., Gu, Y., Ma, H. and Yang, Z. (2013) AtPRK2 promotes ROP1 activation via RopGEFs in the control of polarized pollen tube growth. Mol. Plant **6**, 1187–1201
17 Liu, J., Zhong, S., Guo, X., Hao, L., Wei, X., Huang, Q., Hou, Y., Shi, J., Wang, C., Gu, H. and Qu, L.J. (2013) Membrane-bound RLCKs LIP1 and LIP2 are essential male factors controlling male–female attraction in *Arabidopsis*. Curr. Biol. **23**, 993–998
18 Chae, K. and Lord, E.M. (2011) Pollen tube growth and guidance: roles of small, secreted proteins. Ann. Bot. **108**, 627–636
19 Boisson-Dernier, A., Roy, S., Kritsas, K., Grobei, M.A., Jaciubek, M., Schroeder, J.I. and Grossniklaus, U. (2009) Disruption of the pollen-expressed FERONIA homologs ANXUR1 and ANXUR2 triggers pollen tube discharge. Development **136**, 3279–3288
20 Miyazaki, S., Murata, T., Sakurai-Ozato, N., Kubo, M., Demura, T., Fukuda, H. and Hasebe, M. (2009) ANXUR1 and 2, sister genes to FERONIA/SIRENE, are male factors for coordinated fertilization. Curr. Biol. **19**, 1327–1331
21 Li, S., Ge, F.R., Xu, M., Zhao, X.Y., Huang, G.Q., Zhou, L.Z., Wang, J.G., Kombrink, A., McCormick, S., Zhang, X.S. and Zhang, Y. (2013) *Arabidopsis* COBRA-LIKE 10, a GPI-anchored protein, mediates directional growth of pollen tubes. Plant J. **74**, 486–497
22 Qin, Y., Leydon, A.R., Manziello, A., Pandey, R., Mount, D., Denic, S., Vasic, B., Johnson, M.A. and Palanivelu, R. (2009) Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil. PLoS Genet. **5**, e1000621
23 Leydon, A.R., Beale, K.M., Woroniecka, K., Castner, E., Chen, J., Horgan, C., Palanivelu, R. and Johnson, M.A. (2013) Three MYB transcription factors control pollen tube differentiation required for sperm release. Curr. Biol. **23**, 1209–1214
24 Alexandersson, E., Ali, A., Resjö, S. and Andreasson, E. (2013) Plant secretome proteomics. Front. Plant Sci. **4**, 9
25 Agrawal, G.K., Jwa, N.S., Lebrun, M.H., Job, D. and Rakwal, R. (2010) Plant secretome: unlocking secrets of the secreted proteins. Proteomics **10**, 799–827
26 Drakakaki, G. and Dandekar, A. (2013) Protein secretion: how many secretory routes does a plant cell have? Plant Sci. **203–204**, 74–78
27 Krause, C., Richter, S., Knöll, C. and Jürgens, G. (2013) Plant secretome: from cellular process to biological activity. Biochim. Biophys. Acta **1834**, 2429–2441
28 Kulich, I., Pečenková, T., Sekereš, J., Smetana, O., Fendrych, M., Foissner, I., Höftberger, M. and Zárský, V. (2013) *Arabidopsis* exocyst subcomplex containing subunit EXO70B1 is involved in the autophagy-related transport to the vacuole. Traffic **14**, 1155–1165
29 Murata, D., Nomura, K.H., Dejima, K., Mizuguchi, S., Kawasaki, N., Matsuishi-Nakajima, Y., Ito, S., Gengyo-Ando, K., Kage-Nakadai, E., Mitani, S. and Nomura, K. (2012) GPI-anchor synthesis is indispensable for the germline development of the nematode *Caenorhabditis elegans*. Mol. Biol. Cell **23**, 982–995
30 Lalanne, E., Honys, D., Johnson, A., Borner, G.H., Lilley, K.S., Dupree, P., Grossniklaus, U. and Twell, D. (2004) SETH1 and SETH2, two components of the glycosylphosphatidylinositol anchor biosynthetic pathway, are required for pollen germination and tube growth in *Arabidopsis*. Plant Cell **16**, 229–240
31 Gillmor, C.S., Lukowitz, W., Brininstool, G., Sedbrook, J.C., Hamann, T., Poindexter, P. and Somerville, C. (2005) Glycosylphosphatidylinositol-anchored proteins are required for cell wall synthesis and morphogenesis in *Arabidopsis*. Plant Cell **17**, 1128–1140
32 Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**, 105–132
33 Zimmerman, J.M., Eliezer, N. and Simha, R. (1968) The characterization of amino acid sequences in proteins by statistical methods. J. Theor. Biol. **21**, 170–201
34 Eisenhaber, B., Wildpaner, M., Schultz, C.J., Borner, G.H., Dupree, P. and Eisenhaber, F. (2003) Glycosylphosphatidylinositol lipid anchoring of plant proteins: sensitive prediction from sequence- and genome-wide studies for *Arabidopsis* and rice. Plant Physiol. **133**, 1691–1701

Received 20 September 2013
doi:10.1042/BST20130204

SUPPLEMENTARY ONLINE DATA

In search of ligands and receptors of the pollen tube: the missing link in pollen tube perception

Said Hafidh*, David Potěšil†‡, Jan Fíla*, Jana Feciková*, Věra Čapková*, Zbyněk Zdráhal†‡ and David Honys*[1]

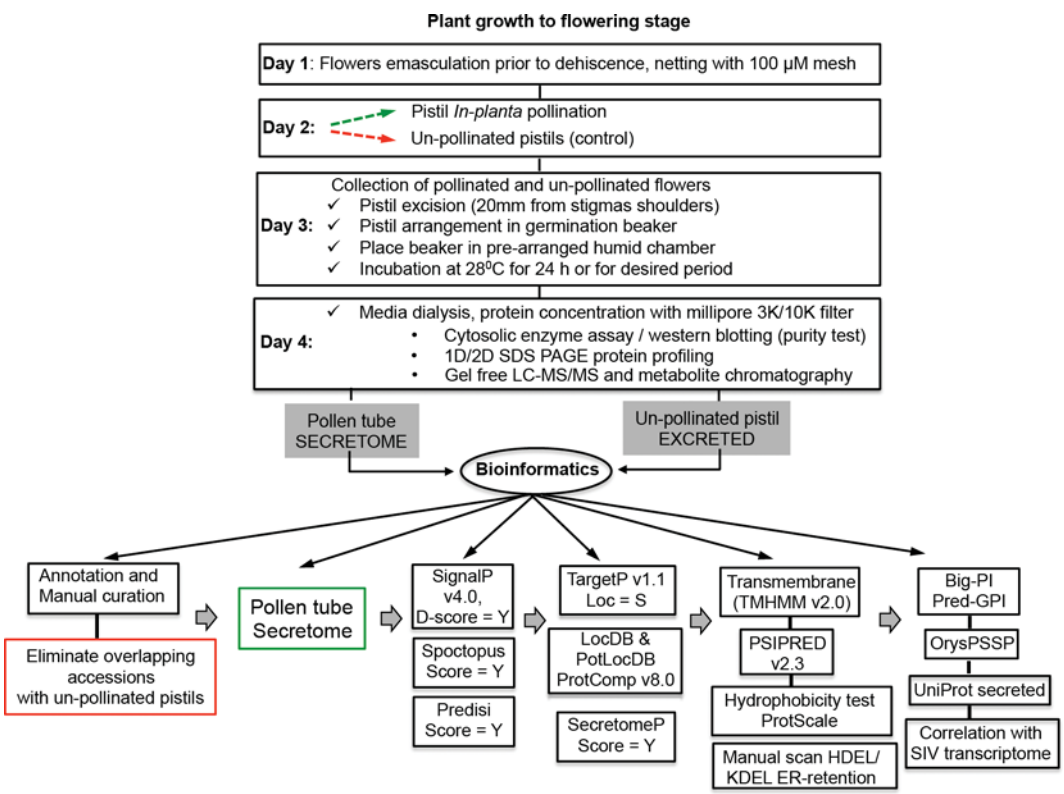
*Laboratory of Pollen Biology, Institute of Experimental Botany ASCR, Rozvojová 263, 165 02 Praha 6, Czech Republic

†CEITEC – Central European Institute of Technology, Masaryk University, Brno, Czech Republic

‡National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, Czech Republic

Figure S1 | **A rough guide towards identification of true secreted proteins following LC–MS/MS**

The tools mentioned here should not replace a manual survey of the data as well as revised databases to assist with the protein annotation and characterization.



Received 20 September 2013
doi:10.1042/BST20130204

[1]To whom correspondence should be addressed (email david@ueb.cas.cz).