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Cell-cell communication during double fertilization

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Double fertilization in flowering seed plants requires intercellular signaling events between many interacting partners. The four cell types of the seven-celled female gametophyte communicate with each other to establish and maintain their identity. They secrete signaling molecules to guide the male gametophyte and to mediate sperm cell discharge and transport towards the two female gametes (the egg and central cell). After fusion of the gametes, guidance signals have to be removed to prevent polyspermy, embryo and endosperm development is induced generating daughter cells or nuclear regions of a different fate, and cell death is induced in the surrounding ovular cells. Until recently, little was known about the molecular nature of the signaling molecules that are involved in these processes. Now, small secreted proteins and peptides have been identified as prime candidates mediating several of these communication events.

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Current Opinion in Plant Biology 2006, 9:41–47

This review comes from a themed issue on
Growth and development
Edited by David Smyth and Thomas Berleth

Available online 1st December 2005

1369-5266/\$ – see front matter
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DOI 10.1016/j.pbi.2005.11.002

Introduction

Double fertilization, a major characteristic of flowering seed plants (angiosperms), was discovered simultaneously by Nawaschin and Guignard [1,2] more than a century ago in the liliaceous plants *Lilium martagon* and *Lilium pyrenaicum* as well as *Fritillaria tenella*. Since this discovery, numerous light and electron microscopic as well as histo-chemical studies have provided a more detailed view of the process [3,4].

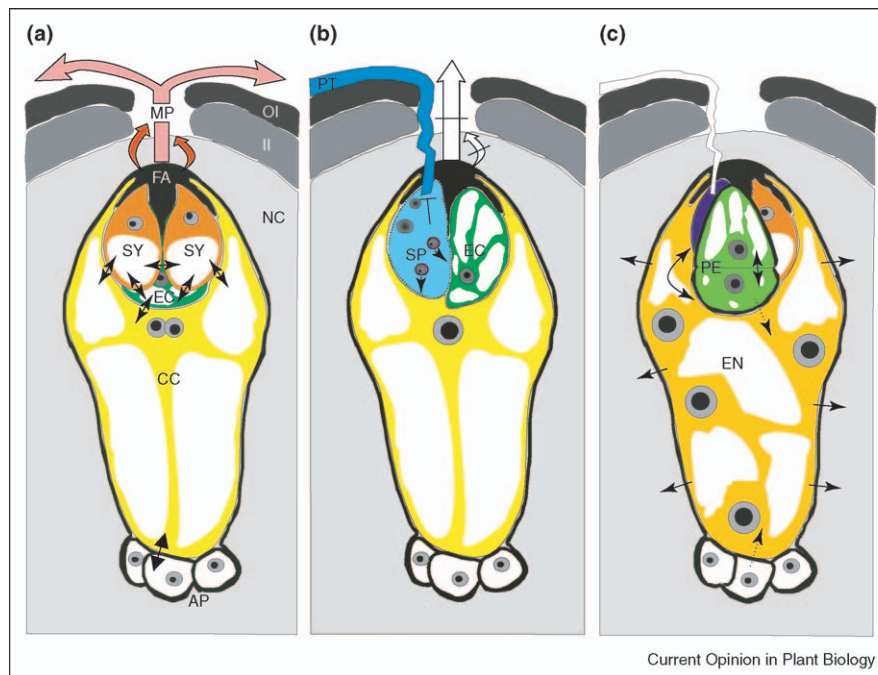
Double fertilization takes place in the female gametophyte (FG), which is also referred to as the embryo sac or megagametophyte. The FG develops from the haploid functional megaspore after three rounds of nucleic mitoses, which generate an eight-nucleate immature FG. During maturation, cells of a different fate are

specified after membrane formation. The three cells at the micropylar pole differentiate into the egg apparatus (EA), which consists of an egg cell that is flanked by two synergids (Figure 1a). The three cells opposite to the EA form antipodal cells, while two nuclei, also referred to as polar nuclei, migrate towards the EA and fuse before or after fertilization to generate the diploid secondary nucleus of the central cell (Figure 1b). The synergids form the filiform apparatus, a complex consisting mainly of cell wall invaginations, and the egg and central cells become highly vacuolated. In plant species such as *Arabidopsis*, the antipodal cells undergo programmed cell death (PCD), whereas in other species, they proliferate, generating a cell cluster of up to 60 cells [5]. In most angiosperms, the haploid mature FG is embedded in several maternal diploid cell layers of the ovule and harbors four cell types, including the two female gametes (the egg cell and central cell) (Figure 1a).

To achieve fertilization, the FG directs the male gametophyte (MG, also referred to as pollen or pollen tube [PT]) towards the EA. The PT penetrates the filiform apparatus, elongates into the cytoplasm of the receptive and degenerated synergid, ruptures and discharges its content, which includes the two sperm cells and the vegetative nucleus (Figure 1b). Two actin 'coronas' are formed in the receptive synergid, and actomyosin-mediated transport of each of the two sperm cells towards one female gamete seems to occur along microfilaments [6,7]. Shortly after the fusion of the gametic membranes and the uptake of sperm cytoplasm by the female gametes, cell wall material is deposited around the fertilized egg cell [8–10]. This material might contribute mechanically to a late block of polyspermy, as does the fertilization envelope in animals. After fusion of the paternal and maternal genomes, the diploid zygote develops into an embryo, while the fertilized central cell forms the triploid endosperm, a tissue that provides nutrition during seed development in dicots and that acts as a storage tissue that is required during germination in many monocots.

The processes described above indicate that many of the most important FG functions involve cell-cell communication events. Intercellular signaling via the apoplast is especially important, as, for example, symplastic connections via plasmodesmata (PD) between the FG and surrounding maternal nucellus cells are absent [11,12], and even these connections become restricted between FG cells during maturation [3,12]. After fertilization, there seems to be no symplastic permeability between the zygote and the primary endosperm: tracers as small as

Figure 1



A generalized model of key cell-cell communication events before and after double fertilization in angiosperms. **(a)** During embryo sac maturation, the seven cells of the female gametophyte (FG) communicate with each other to establish and maintain their identity and position (small double-headed bars). At least two types of molecules are secreted by the egg apparatus to first guide the pollen tube (PT) towards the ovule (ovular guidance, large pink arrows) and afterwards into the micropyle (micropylar guidance, red arrows). In this scheme, the egg cell is positioned behind the synergids, and the central cell still contains two polar nuclei. Vacuoles are drawn in white. **(b)** During or shortly before fertilization, the PT penetrates this synergid and the tip of the PT becomes disrupted and the sperm cells are released. Each sperm cell is guided towards one of the two female gametes. The membranes then fuse, probably due to interactions between cell-surface receptors, and the whole cytoplasm of the sperm cells is generally taken up by the female gametes. Further PT guidance, and thus polyspermy, is probably prevented because of the removal or degradation of the attraction molecules. In this scheme, the persistent synergid is removed and the polar nuclei of the central cell have fused, generating the secondary nucleus. **(c)** Shortly after the fusion of the gametic genomes (karyogamy), the primary endosperm cell initiates repeated rounds of nucleic mitosis. It already contains a few nuclei before the first unequal cell division of the zygote is completed. The two daughter cells, which are now referred to as pro-embryo, have different fates and communicate among each other via the apoplast and/or symplast to establish and maintain their identity. Polarity and cell identity signals are expected to be communicated from the pro-embryo and/or antipodal cells towards the developing endosperm, which itself secretes signaling molecules to induce cell death in the surrounding nucellus cells. Remnants of the degenerated synergid and the PT, as well as the persistent synergid, are still visible. AP, antipodal cells; CC, central cell; EC, egg cell; EN, endosperm; FA, filiform apparatus; II, inner integument; MP, micropyle; NC, nucellus; OI, outer integument; PE, pro-embryo; PT, pollen tube; SP, sperm cells; Sy, synergid.

0.5 kDa could not be transported from endosperm into the zygote [12].

Although double fertilization has been studied extensively at the cytological level in many plant species, surprisingly little knowledge has been generated about the genetic and molecular mechanisms involved [7[•],13,14]. I restrict this review to new approaches and to the recent discovery of (candidate) molecules that are involved in the various cell-cell communication events of double fertilization.

Genetic approaches to uncover molecules that are involved in the fertilization process

In recent years, several genetic segregation and mutant seed set screens (i.e. forward genetic approaches) have been conducted to identify FG mutants in *Arabidopsis*

thaliana [13,15–21]. These screens have identified several genes that encode, for example, a DNA licensing factor [22], a chaperone of the mitochondrial matrix [18], Polycomb group proteins [15,16] and a receptor-like kinase (J-M Escobar, N Huck, U Grossniklaus, pers. comm.). All of these genes are also expressed in tissues other than the FG, however, indicating that they possess more general functions. A large-scale mutant screen of Dissociation (Ds) transposon insertion lines recently identified 130 *Arabidopsis* mutants that have defects in FG development and function, and the corresponding genes. Many mutant lines displayed a defect in embryo sac maturation and polarity (33 lines), whereas fertilization was defective in a smaller number of lines (18 lines). Of the fertilization-defective lines, six were defective in PT attraction, whereas the other 12 mutants showed normal PT attraction but developed unfertilized mature ovules [23^{••}]. The

detailed characterization of the tagged genes might provide us with some yet-unknown molecular key players that are involved in the various FG functions during double fertilization. Genetic screens using plant species such as maize [24] have been less successful and have not resulted in the identification of genes that function in the FG.

A major disadvantage of FG mutant screens is the fact that FG cells are haploid; thus, mutations in housekeeping genes or in genes that are required for general cellular functions, such as cell cycle progression including DNA synthesis and mitosis, could lead to a developmental arrest or dysfunctional nuclear division(s), and thus to female sterility and reduced seed set. Gene redundancy is another problem facing genetic approaches. One member of a gene family might be able to fully or partly complement the downregulation of another member, and therefore many important genes are generally overlooked in these screens [25]. Nevertheless, genetic studies have led to the discovery of several important mechanisms that play a role in the fertilization process. The *magatama* (*maa*) mutant, for example, displays a delayed FG development and thus a loss of PT guidance just before the PT enters the micropyle. In addition, the FG of these mutants frequently attracts two PTs. This led Shimizu and Okada [19] to conclude that the EA probably emits two attractants, one for funicular and one for micropylar guidance, and might also be involved in preventing polyspermy [19]. These genetic studies, together with laser cell ablation studies in *Torenia founieri* [26], led to the sub-division of PT guidance by the FG into funicular and micropylar guidance [19,27].

Two other *Arabidopsis* mutants, *sirene* (*srn*) and *feronia* (*fer*), have PTs that are able to penetrate the filiform apparatus successfully but that fail to burst and to release the sperm cells. These phenotypes indicate that the EA also controls the arrest of PT growth and PT rupture [20,21]. Multiple PTs can enter the embryo sac of *fer* [20], whereas the embryo sac in wildtype plants is penetrated by just one PT, suggesting that the attraction signal is normally rapidly lost or degraded after fertilization. Several studies have been performed to investigate the existence of species-specific or more general guidance signal(s). When *Arabidopsis thaliana* was crossed with pollen of other Brassicaceae species, PTs were able to germinate and grow through the transmitting tissue but not towards the FG, indicating that the FG secretes species-specific signaling molecule(s) [19]. Inter-specific cross-pollination of *Torenia founieri* with related species showed similar results, supporting the hypothesis that species-specific FG signaling exists [27].

In conclusion, it has been estimated that of the thousands of genes that are expressed in the FG, only about 600 can be identified by genetic screens [13], and of these, less

than 10% are involved in the fertilization process [13,23•]. Therefore, alternative methods are required, and the most promising appear to be gene expression-based approaches.

Transcriptomics based approaches and novel markers to study FG signaling

The main advantage of approaches that are based on gene expression (transcriptomics) is the fact that, theoretically, a complete profile of all expressed genes can be obtained. The potential of this approach has been demonstrated recently using the MG of *A. thaliana*. Comparative analysis of the pollen transcriptome using GeneChips® showed that around 7000 genes are expressed in the MG [28,29•]. Interestingly, an unusually high proportion of the proteins encoded by these genes are predicted to be involved in signaling [29•]. Around 5% of the transcribed genes identified by these GeneChips® were specifically regulated during reproductive development in *Arabidopsis* flowers [30•]. Interestingly, many potential signaling molecules, such as receptor-like protein kinases, phosphatases or predicted secreted proteins smaller than 15 kDa, have been identified that could function as signaling molecules or as precursors for peptide hormones.

Cells of the FG can now be isolated from various plant species by micro-dissection [31]. Such isolated cells can be used either to generate cDNAs as probes for micro-arrays or as a source for libraries that are generated from just ~10–20 cells [32•] or fewer [33]. Laser-assisted microdissection (LAM) [34] is expected to increase the availability of cells at certain developmental stages that are not accessible to date, possibly including *Arabidopsis* FG cells. Cells of the maize and wheat FG have been used recently to compare the gene expression profiles of the two female gametes [35], of the egg cell and the two-cell pro-embryo after fertilization [32•], and of the two daughter cells of the zygote [36]. Many of the genes whose expression profiles differ encode candidate signaling proteins. Sperm cells can be isolated in greater numbers using fluorescence-activated cell sorting (FACS). About 8% of the sequences that are expressed preferentially in sperm cells are predicted to encode secreted or plasma-membrane-localized proteins, and therefore might mediate gamete interactions [37].

In summary, during the past two years many expressed sequence tags (ESTs) from tissues that are involved in fertilization have been deposited in the public database. These include more than 5000 ESTs each from sperm and egg cells (S McCormick's laboratory), almost 2200 ESTs from egg cells and pro-embryos (T Dresselhaus laboratory), more than 1000 ESTs from central cells (S Scholten laboratory) and more than 5600 ESTs from whole embryo sacs [38]. A high number of these ESTs are predicted to encode candidate proteins that are

involved in cell–cell communication events and will serve as a source that will help to identify key players that are involved in FG signaling. Reverse genetic approaches are now required to study the functions of candidate genes in detail, a task that will be especially laborious when working with the grass species that provided the majority of available ESTs. Nevertheless, several identified genes display significant homologies to hypothetical proteins in *Arabidopsis* [32*,37], which can be studied instead.

Progress in visualizing the fertilization process has been made thanks to the discovery of several novel markers. Until recently, PT growth has been visualized mainly after staining with either Aniline Blue or Congo Red [39,40]. Pollination with transgenic pollen that expresses markers such as green fluorescent proteins (GFPs) or β -galacturonidase (GUS) (Figure 2a) even in sperm cells [41,42,43**,44*] now makes it possible to follow tube growth and fertilization even in species in which double fertilization occurs deep in the maternal tissues of the ovule. Mostly, unpublished data from various groups have shown that the individual cells of the FG (Figure 2b–d) can be labeled, allowing researchers to study, for example, the establishment and maintenance of their identity.

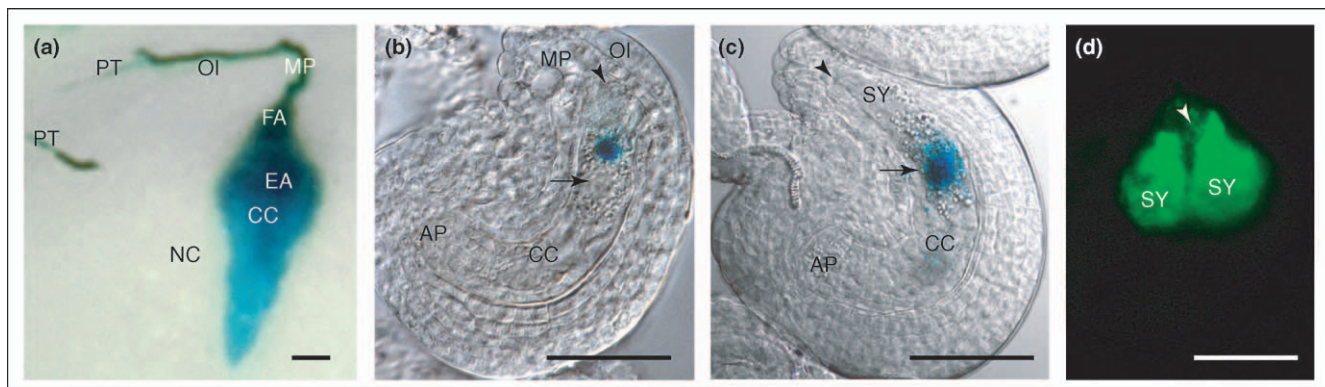
Candidate molecules that are involved in intercellular pre- and post-fertilization signaling

Despite the availability of a large number of ESTs or genes that encode potential signaling molecules that are involved in the processes described above, the functional role of almost all candidate genes is unknown. Sporophy-

tic PT guidance has been shown to be mediated by glycoproteins, gamma-aminobutyric acid (GABA) and small proteins that are secreted in the extracellular matrix (ECM) [39,45]. The latter include Plantacyanin/Chemo-cyanin, a small blue copper protein [46], and the stigma/stylar Cys-rich adhesion protein (SCA) [47]. Small, Cys-rich proteins might also play important roles during fertilization. The maize *ZmES1–4* (*Zea mays EMBRYO SACL1–4*) genes are specifically expressed in the FG and are downregulated shortly after fertilization, suggesting that they have a role in the fertilization process [48]. Before fertilization, *ZmES1–4* proteins accumulate in the synergids and are secreted in the filiform apparatus (Figure 2c). *ZmES1–4* display structural homology to SCA, to the pollen ligand of self-incompatibility (SCR/SP11) in *Brassica*, and to antimicrobial peptides (AMPs). AMPs are small, cationic proteins. They have a characteristic linear arrangement of Cys-pairs that form intramolecular disulfide bridges, and include thionins, defensins, lipid transfer proteins (LTPs), knottins, heveins and snakins [49]. It is very likely that AMPs have functions in processes that are not related to defense, especially during reproduction, as a majority of AMP-encoding genes are expressed during flowering, fertilization and seed development [30*,32*,50,51]. Prominent examples are SCR/SP11 [39,52] and a defensin-like peptide that is required for sperm maturation and storage in mammals [53].

After fertilization, a relatively high number of genes encoding peptides that have structural features of AMPs (e.g. BETL1–4, Basal layer Antifungal Proteins [BAPs],

Figure 2



Markers for the individual cells of the male and female gametophytes are now available, making it possible to visualize the fertilization process. (a) 18 h after pollination, two GUS-expressing PTs are visible at the surface of the outer integument of a maize ovule, but only one penetrates the micropyle, enters the filiform apparatus and discharges its content in the degenerated synergid. The fertilized egg and central cell are stained because of the activation of the paternally derived GUS gene. (b) GUS expression in the mature egg cell and (c) central cell of *Arabidopsis* driven by an egg-cell-specific and central-cell-specific promoter, respectively. The images show whole ovules that contain the FG viewed as in Fig. 1a. The egg cell is localized behind the two synergids. The arrowhead marks the filiform apparatus between the synergids and thus the micropylar tip of the FG. The arrow points towards the secondary nucleus of the central cell surrounded by numerous starch granules. (d) Maize synergids secrete a GFP-labeled peptide into the filiform apparatus (arrowhead). Gene expression is driven by a FG-specific promoter. AP, antipodal cells; CC, central cell; EA, egg apparatus; FA, Filiform apparatus; MP, micropyle; NC, nucellus; OI, outer integument; PT, pollen tube; SP, sperm cells; SY, synergids. The scale bars represent 50 μ m.

and Maternally expressed gene1 [Meg1]) are specifically expressed in the basal endosperm transfer layer of maize [54,55,56^{*}]. The encoded peptides seem to be secreted in the attached placentochalaza region (pedicel) that undergoes PCD after fertilization [57]. Another gene (*ZmESR6*) that encodes a predicted AMP is expressed in the embryo surrounding region (ESR), but the *ZmESR6* protein accumulates in the placentochalaza cells rather than in the ESR cells that produce it [58^{*}]. Some AMPs have been shown to have an *in vitro* inhibitory activity against bacterial and fungal plant pathogens. Nevertheless, it is not clear whether the main function of these peptides is indeed defense against pathogens that arrive with the pollen tube or phloem sap or whether, like the related SCR/SP11 peptide ligand, they act as signaling molecules. The latter hypothesis is supported, for example, by the finding that other *ESR* genes encode small proteins with similarity to the CLAVATA3 meristem signaling molecule [59,60], indicating that the unknown functions of these genes could be signaling rather than defense.

Laser cell ablation studies using the naked FG of *T. foeniculi* have shown that the synergid secretes species-specific diffusible guidance molecule(s) within an effective distance of 100–200 μm . A signaling peptide, which might be degraded after fertilization to prevent polyspermy, has been suggested as a primary candidate for this activity [26,27]. A peptide that has the required characteristics was recently identified in maize [43^{**}]. *ZmEAI* is specifically expressed in the EA, and a EA-GFP fusion peptide was secreted into the cell wall of the EA. From there, the fusion peptide moved into the apoplast of the micropylar nucellus cells towards the surface of the micropyle. After fertilization, *ZmEAI* was no longer detectable and the corresponding gene was downregulated. Transgenic approaches showed that *ZmEAI* is required for close-range micropylar PT guidance, and thus represents the first FG-secreted signaling peptide with a known function. Another small peptide of maize that is probably secreted into the EA cell walls is a novel proteolipid (*ZmTLA*) [61^{*}]. The *ZmTLA* gene is expressed in the EA as well as in the MG. Downregulation of *ZmTLA1* had no effect within the FG but caused defects in the maturation of anther tissues, indicating that it might also have a role during FG maturation.

If the FG-secreted guidance signal(s) are peptides or proteins, it could be expected that these signals would be modified or degraded by extra-cellular proteases after fertilization to prevent, for example, polyspermy (Figure 1b). Several genes that are regulated during or after fertilization and that encode candidate proteases have been reported recently [30^{*},62,63].

Finally, the search for cell-surface molecules that mediate gamete fusion is among the most important challenges for plant reproduction biologists. Recent years have seen the

discovery of several surface proteins that are involved in sperm-egg fusion in animals. These proteins contain at least one transmembrane domain and a large extracellular region for cell-cell adhesion. Carbohydrates seem to mediate cell-specific membrane attachment and fusion is accomplished by fusion proteins that span the intermembrane space, thus physically linking the two membranes [64]. Specific arabinogalactan and other glycoproteins have been detected on the membrane surface of plant sperm cells and female gametes [65–67], and they seem to play a role in FG development [68]. However, the key cell-surface proteins that are required for adhesion and fusion still await discovery.

Conclusions and perspectives

Cell-cell communication is one of the central themes in biology and is of major importance for double fertilization. Thanks to the genetic screens and cell biological approaches described above, the past 15 years have seen a significant increase in information about the various processes involved. Novel, especially transcriptomics-based, approaches have recently uncovered a large number of candidate signaling molecules that might regulate many of the FG functions, including secreted peptide or protein ligands, cell-surface receptors, proteases and kinases. Using marker-expressing lines, it might now be feasible to analyze the transcriptome of labeled FG cells of *Arabidopsis* after FACS or LAM isolation. However, the functions of the various candidate genes remain to be elucidated by reverse genetics and, especially for the grasses, this is the bottle neck in improving our understanding of FG signaling.

Double fertilization was discovered more than a century ago, but we are only just beginning to uncover the molecules that regulate the complex communication of FG cells and their respective functions. However, first identified molecules will now serve as entry points from which to progress the identification of both interacting partners and upstream and downstream molecules. We can expect, therefore, that our knowledge of the fertilization process will enormously increase during the next couple of years.

Acknowledgements

I would like to thank Suseno Amien, Jörg Bantin and Stefanie Sprunck from my lab for the images shown in Figure 2, and Stefanie Sprunck for critical comments on the manuscript. I would like to acknowledge the University of Hamburg for financial support during the past six years.

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