Int. J. Dev. Biol. 49: 615-632 (2005)
doi: 10.1387/ijdb.052023lb

Gametophyte interaction and sexual reproduction: how plants make a zygote

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ABSTRACT The evolutionary success of higher plants relies on a very short gametophytic phase, which underlies the sexual reproduction cycle. Sexual plant reproduction takes place in special organs of the flower: pollen, the male gametophyte, is released from the anthers and then adheres, grows and interacts along various tissues of the female organs, collectively known as the pistil. Finally, it fertilizes the female gametophyte, the embryo sac. Pollen is released as bi or tricellular, highly de-hydrated and presumably containing all the biochemical components and transcripts to germinate. Upon hydration on the female tissues, it develops a cytoplasmic extension, the pollen tube, which is one of the fastest growing cells in nature. Pollen is completely "ready-to-go", but despite this seemingly simple reaction, very complex interactions take place with the female tissues. In higher animals, genetic mechanisms for sex determination establish striking developmental differences between males and females. In contrast, most higher plant species develop both male and female structures within the same flower, allowing self-fertilization. Outcrossing is ensured by self-incompatibility mechanisms, which evolved under precise genetic control, controlling self-recognition and cell-to-cell interaction. Equally important is pollen selection along the female tissues, where interactions between different cell types with inherent signalling properties correspond to check-points to ensure fertilization. Last but not least, pollen-pistil interaction occurs in a way that enables the correct targeting of the pollen tubes to the receptive ovules. In this review, we cover the basic mechanisms underlying sexual plant reproduction, from the structural and cellular determinants, to the most recent genetic advances.

KEY WORDS: *pollen tube, sexual plant reproduction, pollen-stigma interaction, fertilization, cell polarity*

Introduction

The mechanisms of pollination and fertilization were guessed from the practical utilization of agriculture and seed production more than 5000 years ago, when Assyrian priests had rituals of dusting pollen from the male date palms on the inflorescences of female trees (Fig. 1 A,B). The Greek philosopher, Theophrastus (300 B.C) has also inferred on the union of the two sexes for the production of seed in plants. However, the idea was not generalized until 1682, when the botanist Nehemias Grew stated that pollen must reach the stigma to insure the development of seeds. In 1694 Rudolph Jacob Camerarius published «De sexu plantarum epistole», in which he stated that based upon his experiments there are two different parts of the flower, the stamens and the pistil and that they must work together to produce ripe seed. After Brown's (1833) detailed descriptions, it would be Amici (1824, 1830), to firstly observe pollen tubes and its passage down the style and into the ovule (Amici, 1824, 1830, 1847) (Fig. 1 C,D). The contact between the tip of the pollen tube and the egg cell remained unanswered until Strasburger proved that the pollen tube does not stay intact, but its apical tip disintegrates upon contact with the embryo sac and that one of its "nuclei fuses with the nucleus of the egg" (Maheshwari, 1950; Cresti and Linskens, 1999). It was left for Darwin (1862) to prove conclusively the importance of pollination in perpetuation and vigor maintenance of plants. Double fertilization was afterwards simultaneously described by Sergius Nawashin and Leon Guignard in the dawn of the 19th century and nowadays is believed to be a ubiquitous phenomenon (Friedman, 2001).

The male gametophyte in action - the pollen tube

After pollination, pollen is somehow deposited on the stigma surface, where it germinates by re-hydration and development of a fast-growing cytoplasmic extension, the pollen tube. Besides

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0214-6282/2005/$25.00

Printed in Spain
www.intjdevbiol.com

Fig. 1. Historical perspective of the discovery of sexual reproduction in plants. (A,B) *Ancient representations of the first historical evidence that pollen had to be brushed on the stigma surface as a means to assure seed production. As long as 5000 B.C. both Assyrian priests* **(B)** *and Egyptian gods were pictured on ceremonial fertilization of date palms.* **(C,D)** *One landmark of the scientific discovery of the basis of sexual plant reproduction was the precise definition of the pollen tube role in the discharge of the male component made by Giovanni Battista Amici.* **(C)** *shows a wax model by L. Calamai and E. Tortori under the supervision of Amici, illustrating a sagital section of the stigma and style of* Cucurbita pepo *showing the germination and progression of the pollen tube (Amici 1824)(original models are in the Botanical Museum of the University of Firenze, Italy). Amici later moved to explore the especially favourable structural features of orchids.* **(D)** *is reproduced from the original paper from Amici (1847) and shows* camera lucida *drawings of* Orchis morio *(1 to 8, 14, 15),* Orchis mascula *(9),* Orchis abortiva *(nowadays* Limodorum abortivum *)(10-12) and* Orchis maculata *(nowadays* Dactylorhiza maculata *)(13). 1 shows the pollen tubes ("boyaux") reaching the ovaries and* b - *the transmitting tissue. It is remarkable how the author, using the optics and magnification of those days could so clearly distinguish the differences of the pollen tubes and the transmitting tissue cells. 2. Post-fertilized ovule. 3. Next stage of ovule maturation, illustrating the growth of the internal integument. 4. Next stage, with inversion and definition of the mycropyle. 5-8. Fecundation and beginning of embryogenesis. Amici could correctly describe the callose plugs and the discharge in one of the synergids. It took a lot of time and much more sophisticated techniques to see these details with such precision. 9 to 13. Fertilization in other species. 14 and 15, Double embryo formation in* Orchis morio.

their essential role for fertilization, pollen tubes are an excellent model system to study the cellular processes involved in polarity and tip-growth, e.g. cytoskeleton organization, formation and transport of secretory vesicles, endo- and exocytosis, polarized ion gradients and fluxes, periodic behavior and re-orientation of growth (reviewed by Mascarenhas, 1993; Feijó *et al.*, 1995, 2001, 2004; Cheung, 1996b; Hepler *et al.*, 2001). Cytological and molecular studies of these processes are regarded as important both for fundamental studies of fertility control and reproduction in plants and also to elucidate the basic mechanisms in cellular polarization. With limitations some of these could provide templates to explain other tip-growing cells such as fungal hyphae, root hairs or certain specialized animal cells.

Pollen tubes elongate dramatically (up to many centimeters) through the female tissues to deliver the sperm cells into the embryo sac. This growth occurs without further differentiation or division and is restricted to the apical zone, thus depending on the secretion of membrane and cell wall materials at the pollen tube tip (Fig. 2A) (Picton and Steer, 1981, 1983; Lancelle and Hepler, 1992; Roy *et al.*, 1998; Parton *et al.*, 2001). Pollen tubes can be considered one of the fastest growing cells in nature, with rates of up to 4 $\mu m.sec^{-1}$.

Pollen tubes are intrinsically polarized cells, a feature reflected in an internal differentiation defining distinct intracellular zones (Cresti *et al.*, 1977; Uwate and Lin, 1980; Lancelle and Hepler, 1992; Hepler *et al.*, 2001): (A) the tip domain, rich in Golgi secretory vesicles outlining an inverted cone; (B) the sub-apical domain, enriched in a large population of metabolically active organelles, such as mitochondria, dictyosomes, ER, vesicles and a few large organelles; zones A and B are usually referred to as the "clear zone"; (C) a nuclear domain, containing most of the large organelles and the male germ unit and further back (D) the vacuolar domain, containing a large vacuole which enlarges as the tubes grow (. 2 a-b). Zones C and D show a distinct "reverse fountain" streaming pattern, with organelles moving as fast as 10 $\mu m.sec^{-1}$. A callose plug isolates the growing part from the remainder of the tube, which eventually dies. Thus, just the front part of the tube is alive and growing.

An important feature of a growing pollen tube is the "reverse fountain" cytoplasmic streaming, in which an ordered forward movement of organelles through the cortical region of the tube undergoes a turnover in the subapical domain moving back centrally, away from the tip of the tube (Iwanami, 1956). Several studies demonstrated the involvement of the actin cytoskeleton in cytoplasmic streaming and the transport of secretory vesicles containing cell wall precursors (Steer and Steer, 1989; Pierson and Cresti, 1992; Derksen *et al.*, 1995; Li *et al.*, 1997). The actin microfilaments are organized in small cables or a meshwork of filaments (Fig. 2C) and are believed to be regulated by a dynamic process of polymerization/ de-polymerization (Lancelle and Hepler, 1992; Miller *et al.*, 1996; Vidali *et al.*, 2001; Feijó *et al.*, 2004). The apical region is essentially devoid of organized filaments, but a mesh of very short and highly dynamic fragments are observed in the subapical region and in less amount in the "clear zone" (Fu *et al.*, 2001; Chen *et al.*, 2002), where vesicles present apparently random brownian-like motion (Pierson *et al.*, 1990). Several genes encoding isoforms of actin expressed in mature pollen grains have been identified (Thangavelu *et al.*, 1993; An *et al.*, 1996; Huang *et al.,* 1996; Huang *et al.*, 1997). During pollen maturation there is a switch from vegetative to reproductive actin isoforms, which are believed to fulfill unique functions during pollen development (Kandasamy *et al.*, 1999).

In recent years proteins that influence cell shape by regulating the organization and polymerization of cytoskeletal filaments were described by different groups. Several actin binding proteins such as profilin, villin and ADF/cofilin act as regulators of actin cytoskeleton assembly and have been shown to play active roles in the control of the differential distribution of organelles and vesicles and in proper growth of the pollen tube (Kim *et al.*, 1993; Staiger *et al.*, 1993; Lopez *et al.*, 1996; Vidali and Hepler, 1997; Vidali *et al.*, 1999; Smertenko *et al.*, 2001; Allwood *et al.*, 2002; Chen *et al.*, 2002; Hussey *et al.*, 2002a; McKenna *et al.*, 2004).

Contrasting with our current knowledge about actin filaments, the role microtubules play in pollen development and tube growth remains unclear. Microtubules are made up of tubulin and are known to interact with microtubule associated proteins (MAP)(Hussey *et al.*, 2002b). Microtubules were proposed to be involved in the regulation of pollen tube morphogenesis and in the maintenance of internal cytoplasmic organization, but not in active pollen tube growth (Joos *et al.*, 1994; Derksen *et al.*, 1995). They also have been shown to regulate the position of the male germ unit (Sorri *et al.*, 1996; Rodriguez-Garcia *et al.*, 2003). Microtubule motor proteins have been isolated in pollen tubes, but their functions are mostly unknown (Cai *et al.*, 1996). The *zwichel* (*swi*) mutation, known to affect trichome branching, encodes a mutant kinesin-like calmodulin-binding protein (KCBP). The *suz1* and *suz3* (suppressor of *zwi3*) mutants display no phenotype in absence of *zwi3* mutation, but interestingly, the double mutant *suz1 zwi3*, while rescuing the branch defects, is male sterile due to a defect in pollen germination and tube growth. The few pollen tubes that elongate show large spherical bodies, probably vesicles, suggesting that *suz1* or *swi* could have functions on vesicle movement or targeting during pollen tube growth (Krishnakumar and Oppenheimer, 1999).

Recently, an *Arabidopsis* AFH1 formin was expressed in pollen tubes and shown to be important for maintaining tip-focused cell membrane expansion. The authors suggest that formins located at the cell surface may be mediators of external stimuli to the actin cytoskeleton and thus, AFH1 could be important for mediating the extracellular signals from female tissues and to elicit the proper pollen tube growth response during pollination (Cheung and Wu, 2004, review in Feijó *et al.*, 2004).

In yeast small GTPases control polarity establishment, cytoskeletal organization, polar secretion and cell wall assembly. Recent experimental data provided evidence that a Rop (Rho–related GTPases from pollen) plays a critical role in coordinating pollen tube growth, probably by modulating tip Ca^{2+} gradients and actin dynamics (reviewed by Zheng and Yang, 2000; Cheung *et al.*, 2003; Gu *et al.*, 2003). Both immunolocalization and GFP-tagging showed that Rop is localized to the apical domain of the plasma membrane of pollen tubes, probably acting to focus secretory vesicle delivery (Lin *et al.*, 1996; Lin and Yang, 1997; Kost *et al.*, 1999; Li *et al.*, 1999; Wu *et al.*, 2001). Expression of dominant negative forms of Rac/Rop GTPases resulted in reduced actin bundling and inhibited pollen tube growth, whereas their overexpression converted polar growth into isotropic growth, resulting in pollen tubes with balloon tips and a disrupted actin cytoskeleton (Kost *et al.*, 1999; Li *et al.*, 1999). Using the Rac/Rop

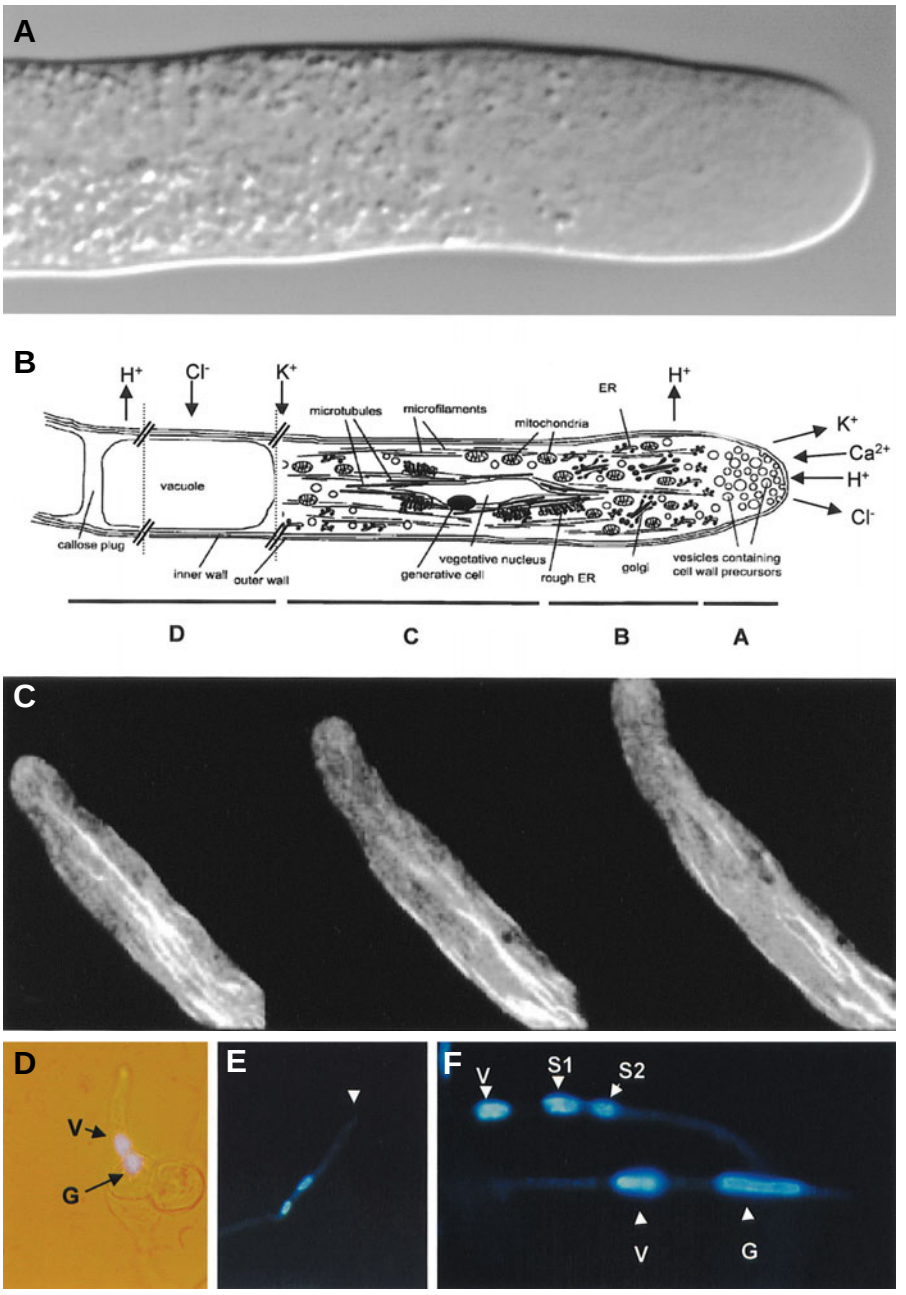


Fig. 2. The male partner – the pollen tube. (A) *DIC image of a* Lilium longiflorum *pollen tube (diameter ~ 15 μm). The polarisation of the growing tip is striking; the growing apex is devoid of large organelles and presents no organised movement (the "clear zone"). Behind it all the larger organelles are sorted out and move backwards on a fast, organised reverse-fountain streaming pattern. In this species tubes can grow* in vitro *as fast as 20 μm.min*$^{-1}$. **(B)** *Diagrammatic representation of a pollen tube.* **(A-C)** *Cytoplasmic domains;* **(A)** *Clear zone;* **(B)** *subapical domain;* **(C)** *nuclear domain;* **(D)** *vacuolar domain containing first callose plug (relative length of the domains is not drawn to proportion). Arrows represent ion fluxes known to be important on the establishment of polarity and pollen tube growth. For details see text. Adapted from Franklin-Tong (1999).* **(C)** *Time course analysis of growth of a* Nicotiana tabacum *pollen tube transiently expressing a fusion protein construct of LAT52 (pollen specific promoter), ADF (actin-depolymerising factor) and GFP, imaged with two-photon microscopy. The 3 frames are separated by 30 seconds each. Growth of the tube is evident, as well as the fast dynamics of the actin cytoskeleton in the formation and constant reshaping of the fibrils along the pollen tube. A finer meshwork of smaller filaments is present in the subapical area, but this sequence was contrast-optimised to see the long actin filaments that promote the vigorous streaming so characteristic of these cells. (Bar: 10 μm) (adapted from Feijó and Moreno, 2004).* **(D-F)** *Sequence of the nuclear migration and division of the generative cell during pollen germination of* O.lutea. *Both generative cell* **(G)** *and vegetative nuclei (V- in front) enter the pollen tube soon after germination* **(D)**. *This male germ unit travels at a relatively fixed position form the tip (arrow)* **(E)**. *Finally, at a timing corresponding to the entrance of the ovule when germinated* in situ, *the generative cell elongates (bottom tube) and divides into the two sperm cells (S1 and S2- top tube) (nuclei labelled with DAPI)(adapted from Feijó, 1995).*

GTPase-induced defective pollen tube phenotype as a functional assay, Chen *et al.* (2003) have shown that overexpression of *NtADF1* suppresses the ability of NtRac1, a tobacco Rac/Rop GTPase, to convert pollen tube tip growth to isotropic growth. This finding suggests that NtADF1 (*Nicotiana* actin depolymerizing factor1) acts in a common pathway with NtRac1 to regulate pollen tube growth. The mechanisms of docking and fusion of vesicles and the molecular events underlying these processes are not yet understood, but the dynamics of membrane flux was recently addressed by using lipophilic dyes, such as FM4-64 (Parton *et al.*, 2001; Camacho and Malhó, 2003; Parton *et al.*, 2003). The results indicate a dynamic and intrinsically regulated process of endo/exocytosis present at the tip of the pollen tube, independent of pollen tube growth (Parton *et al.*, 2003). The GFP-NtRab2 fusion protein was shown to function in the secretory pathway between the endoplasmic reticulum and the Golgi suggesting that tip growth and vesicle trafficking are tightly linked in elongating pollen tubes (Cheung *et al.*, 2002). Moreover, two male gametophytic mutants, *pok* (*poky pollen tube*) and *kinky pollen* (*kip*) show aberrant and reduced pollen tube growth and are known to encode proteins involved in vesicle trafficking (Procissi *et al.*, 2001; Lobstein *et al.*, 2003; Procissi *et al.*, 2003; Lobstein *et al.*, 2004).

The pollen tube wall is differentiated between the primary wall, secreted in the growing tube tip and containing mainly pectin, a middle hemicellulosic layer and an inner secondary wall deposited behind the growing tip, that contains mainly callose (Heslop-Harrison, 1987; Derksen *et al.*, 1995). The pectin layer is expected to control the wall dynamics at the growing tip, being plastic enough

to allow cell extension but also strong enough to support the high hydrostatic pressure exerted by the pollen tube cytoplasm (Steer and Steer, 1989; Benkert *et al.*, 1997; Holdaway-Clarke *et al.*, 2003; Geitmann and Parre, 2004). The finding that unesterified and methyl-esterified pectin epitopes localize in the tip of the wall supports the hypothesis that pectins are polymerized and esterified within the Golgi complex and then transported and deposited in the growing wall by secretory vesicles (Li *et al.*, 1995; Li *et al.*, 1997). After deposition, the esterified pectins become progressively de-esterified by the action of pectin methyltransferases (PME) leading to a more rigid form of pectin in the mature pollen wall by crosslink with Ca^{2+} ions and boric acid (Carpita and Gibeaut, 1993; Li *et al.*, 1997; Li *et al.*, 2002; Holdaway-Clarke *et al.*, 2003). The deposition of the secondary wall starts later and increases gradually in thickness back from the tip. Enzyme complexes are presumably responsible for the production of two types of beta-glucan, the 1,3-β-glucan callose and the 1,4-β-glucan cellulose (Ferguson *et al.*, 1998). In *Nicotiana* both a cellulose–synthase and a glucan-synthase gene have been identified and shown to be predominantly expressed in the male gametophyte. They probably encode catalytic subunits of two β-glucan synthases involved in pollen-tube wall synthesis (Doblin *et al.*, 2001). In lily two exo-β-glucanases were also shown to be expressed in pollen tubes and may be involved in the regulation of pollen tube elongation by hydrolyzing callose and 1,3:1,4-β-glucan within the pollen tube walls (Takeda *et al.*, 2004).

Besides callose and cellulose, several hidroxyproline-rich glycoproteins (HRGPs) have been reported to be present in the secondary tube wall (Cassab, 1998; Showalter, 2001). Examples of these glycoproteins are the pollen-specific extensin-like protein (Pex1) of maize and the pollen–specific glycoprotein NTP303 of tobacco (Weterings *et al.*, 1992; Rubinstein *et al.*, 1995; Wittink *et al.*, 2000). The presence of a conserved leucine-rich repeat (LRR) in the known Pex proteins strongly suggests that this motif is involved in the binding of a specific ligand during pollen tube growth (Stratford *et al.*, 2001). Arabinogalactan-proteins (AGPs) were also identified in the pollen tube walls and thought to have an important role during pollen tube growth. Pollen tubes treated with Yariv reagent, which crosslinks with AGPs are arrested, suggesting a direct role of AGPs in tip growth in monocot species (Li *et al.*, 1995; Jauh and Lord, 1996; Roy *et al.*, 1998; Mollet *et al.*, 2002).

Crucial for pollen tube function is also the presence of membranar receptors for external female stimuli. Several pollen receptor-like kinases (PRKs) of tomato and petunia have an extracellular domain including LRRs, transmembrane domains and intracellular domains and thus are good candidates to play a role in pollen–style recognition. Some of these proteins were recently shown to localize to the pollen tube wall and presumably interact with female ligands (Mu *et al.*, 1994a; Muschietti *et al.*, 1998; Skirpan *et al.*, 2001; Kim *et al.*, 2002; Wengier *et al.*, 2003; Tang *et al.*, 2004). Glycosylphosphatidylinositol (GPI) anchoring provides an alternative to transmembrane domains for anchoring proteins to the cell surface and thus provide another alternative to male-female interaction. Recently, two mutations disrupting *SETH1* and *SETH2*, which encode *Arabidopsis* homologs of two conserved proteins involved in the first step of the GPI biosynthetic pathway, presented reduced pollen germination and tube growth, associated with abnormal callose deposition (Lalanne *et al.*, 2004). The authors also identified 47 genes that encode potential GPI-anchored proteins expressed in pollen. Most of the identified proteins show homology with proteins involved in cell wall synthesis and remodeling or intercellular signaling and adhesion and thus are likely to play an important role in pollen tube polarization.

Pollen tubes were also the focus of a considerable interest due to the oscillatory nature of their growth, first observed by the periodic formation of thickened cell wall rings occurring in some species, later correlated to fluctuations in the growth rate (review in Li *et al.*, 1997). While the plasma membrane appears to have a smooth continuous membrane underlying the cell wall, it contains ion channels thought to follow a polarized distribution/activation that define the growth axis and also the growing shape (reviewed Feijó *et al.*, 1995, 2001; Holdaway-Clarke and Hepler, 2003). The use of non-invasive methods, such as cytosolic ion concentration imaging, ion vibrating probes and patch clamp have been intensively explored to characterize the physiology of pollen tube growth. It is known for long that pollen tube growth is a Ca^{2+} dependent process (Brewbaker and Kwack, 1963). Pollen tubes possess a localized gradient of cytosolic free calcium at their apex, which is associated with an extracellular Ca^{2+} influx (Rathore *et al.*, 1991; Miller *et al.*, 1992; Pierson *et al.*, 1996; Holdaway-Clarke and Hepler, 2003). If the Ca^{2+} gradient is disrupted, growth ceases in correlation with the dissipation of the gradient. The extracellular influxes of Ca^{2+} were found to be restricted to the very tip of the pollen tube and both the cytosolic Ca^{2+} gradient and the inward Ca^{2+} current were shown to have an oscillatory mechanism that correlates with growth oscillations (Holdaway-Clarke *et al.*, 1997; Messerli and Robinson, 1997; Feijó *et al.*, 2001, Holdaway-Clarke and Hepler, 2003). It is generally accepted that the calcium gradient is maintained by the existence of stretch-activated Ca^{2+} channels underlying the influx at the pollen tube tip, putatively described by patch clamp on pollen protoplasts by Dutta and Robinson (2004). Analysis of the family of autoinhibited Ca^{2+} ATPases (*ACA*) in *Arabidopsis*, showed that the isoform *ACA9* was pollen-specific and its expression was localized at the plasma membrane along all the tube (Schiott *et al.*, 2004). Gene disruptions of the *ACA9* coding sequence were found to result in partial male sterility. The *aca9* mutant pollen displayed a reduced tube growth potential and a high frequency of aborted fertilization, suggesting that this channel is a regulator of pollen development and fertilization in flowering plants.

Chloride is one of the ions involved in the regulation of pollen tube growth. Large oscillatory extracellular fluxes are closely coupled to oscillatory growth and to the cell volume of the apical domain in the pollen tube tip. The role of chloride effluxes is suggested to be essential for pollen tube to regulate cell volume or turgor pressure (Zonia *et al.*, 2001).

Potassium has been suggested to function as membrane potential regulator and recently a pollen-specific inward K^+ channel belonging to the Shaker family was isolated. The analysis of *spik* heterozygous plants revealed impaired pollen tube growth and reduced competitive ability (Mouline *et al.*, 2002).

Cytosolic pH is known to modulate cytoskeleton dynamics, vesicle exocytosis and enzymatic activity in many developmental processes. In pollen tubes a proton influx is localized in the extreme apex, that decreases in the subapical region reverting to an efflux (Feijó *et al.*, 1999). This pattern of fluxes correlates with an intracellular acidic tip and a constitutive alkaline area described at the base of the apical region. Oscillations were shown to correlate

with growth and extracellular fluxes both at the level of the alkaline area and on the acidification at the tip; protons are believed to enter passively or through low affinity cationic channels at the apex and to be extruded at the subapical domain through plasma membrane H^+-ATPases (Feijó *et al.*, 1999). Despite the effort to unveil the major mechanistic components of pollen tube growth regulation, integrative models of their regulation linking ion dynamics, cell wall extension and the intracellular organization remain mostly speculative (Feijó *et al.*, 2001, 2004).

The progamic phase

The progamic phase corresponds to the progression of the pollen tube through the gynoecium, beginning with germination of the pollen grain and penetration of the pollen tube in the stigma and culminating with the delivery of the sperm cells into the synergid of the embryo sac (Fig. 3A, I-III). When pollen tubes grow on the pistil, they follow invariably the same path. For this reason the pistil was seen for long as a passive structure, just providing nutrients needed for pollen tube growth. However, recent evidence shows a different view and it is now commonly accepted that the pistil plays an active role, perhaps even acting dynamically on the regulation of pollen tube growth. Most of the current knowledge involving the events occurring after contact of pollen with the stigmatic surface was gathered from studies on the self-incompatibility system in several species. Yet substantial information is also available on cell-to-cell communication events and on the molecules involved in compatible crosses (de Graaf *et al.*, 2001; Wheeler *et al.*, 2001; Johnson and Preuss, 2002; Higashiyama *et al.*, 2003; Lord, 2003).

Pollen-stigma interactions

The stigma can be considered a modified structure for pollen grain capture and reception, germination and initial growth of pollen tubes. The stigmatic surface of different species varies widely in morphology and in the presence or absence of stigmatic exudates. There are two types of stigma, the “dry” and the “wet” types, which differ by the presence or absence of wet sticky secretion, the exudate. It was suggested that in plants with “dry” stigma the pollen coat assumes the role of the exudate present in plants with “wet” stigmas (Heslop-Harrison and Shivanna, 1977; Knox, 1984). The initial contact between the male pollen and female stigma cells includes specific and selective binding of appropriate pollen by the stigma, rapid pollen-recognition, pollen hydration and pollen tube germination and growth.

Crucifers, like *Brassica* and *Arabidopsis,* have a “dry” stigma and the epidermis is composed of large specialized papillae cells that interact directly with the surface of pollen. Genetic and chemical papillar cell ablations in *Brassica* and *Arabidopsis* led to different results, despite the fact that the processes leading to pollen recognition, hydration and germination appear to be similar. In *Brassica* the impairment on the biosynthetic activity of papillar cells led to inhibition of pollen tube development, whereas in *Arabidopsis* pollen tubes were able to germinate and grow in non functional stigmas (Kandasamy *et al.*, 1993; Thorsness *et al.*, 1993).

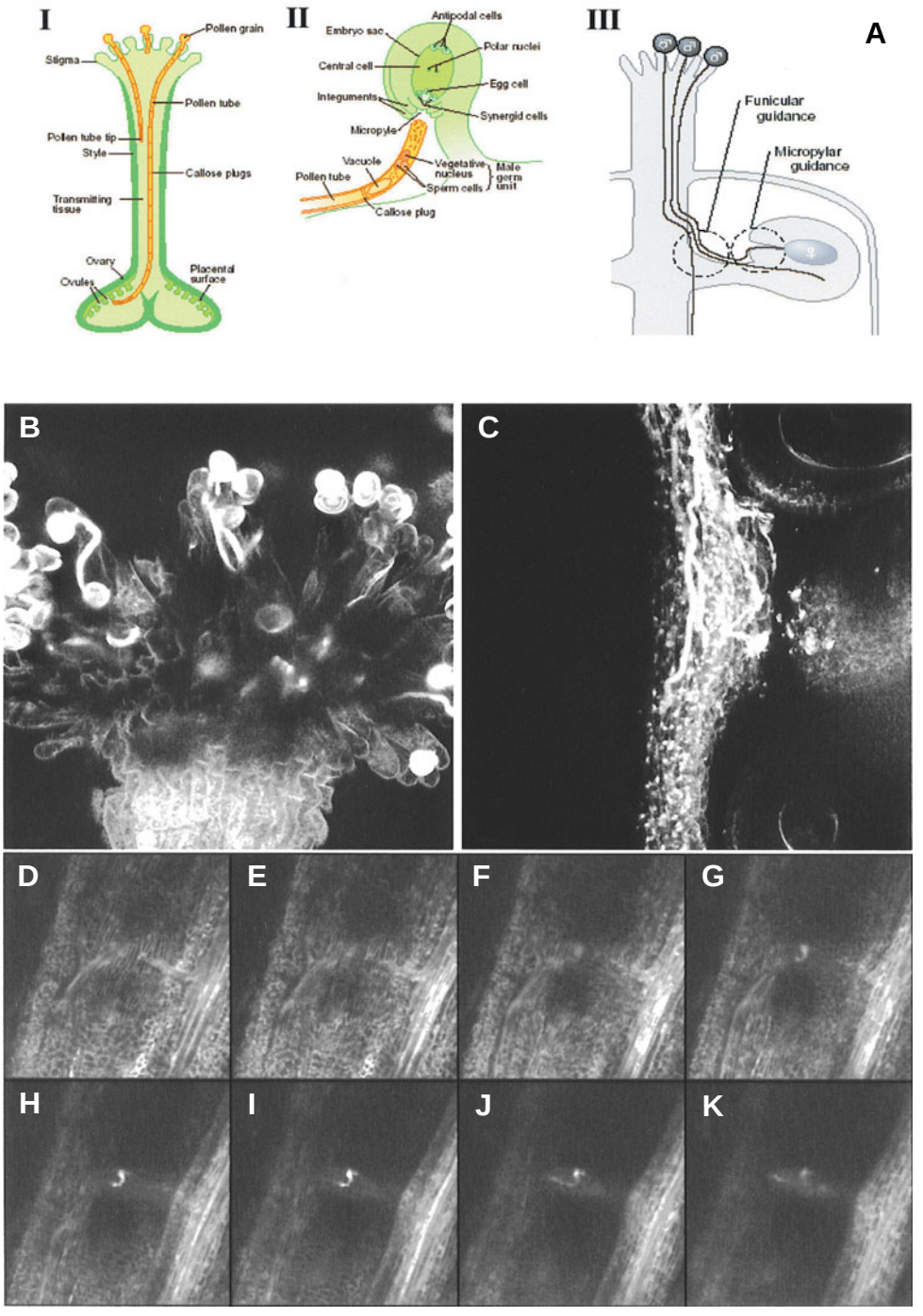
The first event known to occur when pollen lands on plants with “dry” stigmas is pollen adhesion. Although the biophysical and chemical nature of the adhesion process are unknown it is thought to depend on the deposition and mobilization of components of the pollen coat to the stigmatic surface and is likely to involve also components of the stigmatic surface to promote the formation of adhesion bonds (Heslop-Harrison and Shivanna, 1977; Elleman *et al.*, 1992).

In *Brassica oleracea* quantitative measurements of the pollen-stigma adhesion demonstrated that the nature of the interface contact undergoes structural changes that are correlated with reinforcement of the adhesion contact over time (~30 min). Pollen coating is one of the major components of this interaction. On the female side, the developmental stage of the stigma and the protein constituents of the stigmatic cuticle are critical for pollen capture, whereas the SLG and SLR1 glycoproteins (involved in self-incompatibility) play a role in later stages (Luu *et al.*, 1997a, 1997b, 1999). Pollen lipids and stigmatic waxes are believed to take part in the formation of a contact interface between pollen and stigma (Heslop-Harrison and Shivanna, 1977; Elleman *et al.*, 1992; Preuss *et al.*, 1993). In *Arabidopsis* a very rapid “initial” adhesion step was demonstrated to rely on the exine coat, but not on the pollen coat (Zinkl *et al.*, 1999). Several mutants showing defects in exine are also defective for adhesion to papillae (Taylor *et al.*, 1998; Zinkl and Preuss, 2000; Paxson-Sowders *et al.*, 2001; Ariizumi *et al.*, 2003).

To date one of the best described signal transduction mechanism in pollen-pistil interactions is the process of recognition in the sporophytic self-incompatibility (SSI) system in *Brassica* (Schopfer *et al.*, 1999; Kachroo *et al.*, 2001; Takayama *et al.*, 2001). It involves the pollen coat S-locus cysteine-rich protein SCR (also designated SP11) and two stigmatic proteins SLR1 (S-locus related protein) and SLG (S-locus glycoprotein) (Luu *et al.*, 1999; Kachroo *et al.*, 2001; Takayama *et al.*, 2001). Several small cysteine-rich pollen coat proteins (PCPs) were shown to interact with SLG and SLR1 (Doughty *et al.*, 1993; Hiscock *et al.*, 1995; Doughty *et al.*, 2000). Pollen grains that are rejected fail to hydrate on the stigmatic surface (Luu *et al.*, 1999; Doughty *et al.*, 2000; Takayama *et al.*, 2000; Shiba *et al.*, 2001; Takayama and Isogai, 2003).

Recent evidence demonstrates that ligand-receptor kinase signals may be involved in mediating pollen-pistil interactions in compatible pollinations. Two pollen specific receptor kinases LePRK1 and LePRK2 were shown to localize to the pollen tube wall and to associate forming a 400-kDa protein complex. The LAT52 cysteine-rich protein, known to be important in pollen germination and tube growth, interacts with the extracellular domain of the LePRK2 just before germination. One of the female components, LeSTIG1, a pistil specific cysteine-rich protein, binds to the extracellular domains of LePRK1 and LePRK2, displacing binding of LAT52 *in vitro*. Further components of this signaling pathway are expected to reveal important cell-to-cell communication events leading to pollen tube growth on the female tissues (Muschietti *et al.*, 1998; Kim *et al.*, 2002; Tang *et al.*, 2002; Wengier *et al.*, 2003; McCormick, 2004; Tang *et al.*, 2004).

After the process of recognition and compatible pollination the pollen hydrates and germinates. Although *in vitro* pollen germination is quite fast, the *in vivo* timing varies greatly depending on the species, from 15 minutes in *Arabidopsis* to more than 60 minutes in *Brassica oleraceae* (Preuss *et al.*, 1993). Aquaporins have been suggested for the transcellular and symplastic selective transport of water and putative candidates to control pollen hydration on the stigma (Tyerman *et al.*, 2002). The screening of an anther cDNA



library in *Brassica* led to the isolation of two clones, *mipA* and *mipB*, which show high similarity with aquaporins. These proteins are probably targeted to the pollen coat (Ruiter *et al.*, 1997). The recessive mutation *mod* in *Brassica* encodes an aquaporin and results in the breakdown of the self-incompatible reaction. The water channel was proposed to be a component of the SSI signaling pathway (Ikeda *et al.*, 1997). Moreover, the isolation of a gene, *MIP-MOD*, linked to the *MOD* locus and encoding an aquaporin-like protein, increased the expectations that this type of channels may be involved in the regulation of water transport from the stigma papilla to the pollen grains (Dixit *et al.*, 2001).

Several mutants were identified that showed spatial or temporal disturbances in pollen water control. In *raring-to-go* mutant plants pollen grains acquire or retain water from the anther, germinating precociously and thus circumventing the required contact with the female stigma (Johnson and McCormick, 2001). *Fiddlehead (fdh)* shows defects in organ fusion, and wildtype *Arabidopsis* pollen grains are able to adhere, germinate and produce pollen tubes when applied to vegetative and non reproductive floral organs (Lolle and Cheung, 1993; Lolle *et al.*, 1998). The gene is known to encode a protein showing similarity to a large class of genes that encode proteins related to beta-ketoacyl-CoA synthases and chalcone synthases. The gene may have a role in the biosynthesis of long-chain lipid components that are localized extracellularly, probably modifying the properties of the cuticle (Pruitt *et al.*, 2000). In *Arabidopsis* mutants that belong to the *eceriferum* (*cer*) family with impaired long-chain lipid biosynthesis, the pollen coat composition

Fig. 3. Pollen tube guidance on the pistil. A(I) *In a typical compatible pollination, pollen adheres and germinates on the stigma producing a pollen tube, which grows through the female tissues towards the ovary,* **A(II)** *where pollen tubes have to adjust their growth trajectory to find an ovule and then turn again to enter in the mycropyle penetrating the embryo sac. Adapted from Cheung and Wu (2001).* **A(III)** *Current model for pollen tube guidance involves a funiculus guidance signal that leads the pollen tube to exit the transmitting tissue and a micropylar guidance signal which changes the direction of pollen tubes to the mycropyle. Male-male repulsion, a blocking signal, or cessation of the attracting signal may prevent polyspermy. Adapted from Higashiyama* et al. *(2003).* **(B,C)** *Pollen germination on the stigma and growth through the style and ovary, observed on living material by two-photon imaging of LAT52-GFP* Arabidopsis *pollen tubes. Pollen tubes grow along and around the stigmatic papillae until penetration of the style* **(B)** *and then move along the transmitting tissue in bundles* **(C)**. **(D-K)** *Optical sectioning through an intact living ovary of* A. thaliana, *fertilized with pollen transformed with a fusion product of the pollen specific promoter LAT52 and GFP. The whole plant was brought intact into the microscope and the flower bent into a drop of water on top of a Fluor water dip objective lens (60x; NA, 1.0; working distance, 2.0 mm). Selected views from a Z-stack made from the external surface of the ovary, past through the epidermis, the ovary locus and a ovule along which a pollen tube crawls into the micropyle. (Bar, 100 μm)(adapted from Feijó and Moreno, 2004).*

is also affected and plants are male sterile, showing severe reduction in the ability to hydrate on the stigma (Preuss *et al.*, 1993; Hulskamp *et al.,* 1995a; Zinkl *et al.*, 1999). Experiments involving the application of exogenous purified lipids to *cer* mutants were able to bypass pollen hydration specificity and induced heterologous grains to hydrate and germinate pollen tubes (Zinkl and Preuss, 2000).

In species with "wet" stigmas, such as tobacco, the secreting exudate compounds are involved in a variety of processes such as pollen capture, pollen recognition and control of water availability for proper pollen germination. The lipid fraction of the exudates contains many saturated and unsaturated fatty acids, which usually occur as triacylglycerides. Other major components are phenols, proteins, enzymes, glycoproteins and polysaccharides (Cresti *et al.*, 1986). Mutations that eliminate the exudate from stigmas are known to cause female sterility (Goldman *et al.*, 1994; Wolters-Arts *et al.*, 1998). In transgenic stigmaless plants the addition of purified triacylglycerides restored the ability of pollen to penetrate the stigma or other vegetative surfaces, e.g. leaves (Wolters-Arts *et al.*, 1998). Moreover, different lipids have been shown to have effects on pollen grain hydration time, suggesting that lipids may interact with the pollen grain surface (exine, intine, plasma membrane) to change its permeability to water (Lolle *et al.*, 1997; Lolle and Pruitt, 1999; Pruitt *et al.*, 2000; Wolters-Arts *et al.*, 2002). Pollen coat lipids are present in several *Brassicaceae* species and it is thus less conceivable that they could act as signal molecules to confer species specificity (Piffanelli *et al.*, 1998; Edlund *et al.*, 2004). In contrast to pollen coat lipids, glycine rich proteins (GRPs) are highly divergent across *Brassicaceae* and thus are best candidates to mediate pollen recognition in a species specific manner (Mayfield *et al.*, 2001; Fiebig *et al.*, 2004).

During hydration the pollen grain has to re-organize the plasma membrane and its cytoplasm in such a way that it restores the metabolic activity and competence to establish a polarity axis leading to the production of a pollen tube that emerges from one of the pollen grain apertures (Heslop-Harrison, 1987; Tiwari and Polito, 1990; Mazina *et al.*, 2002). The signal that determines the place of pollen tube emergency is not known, although water and lipids have been suggested to provide the directional cues that establish polarity (Lush *et al.*, 1998; Wolters-Arts *et al.*, 1998).

Pollen germination is known to be a density dependent process, the so-called "mentor" effect. A small peptide, phytosulfokine-α, was shown to promote pollen germination cultured at low density (Chen *et al.*, 2000). Phytosulfokine makes part of a small group of peptides known to have a role in cell-cell communication via receptors (Matsubayashi *et al.*, 2001; Wisniewska *et al.*, 2003).

After pollen germination the style is penetrated and the pollen tube either grows through the papilla cell wall in plants of dry stigmas (Fig. 3B) or is directed through the intercellular spaces between the cells of the secretory region in species with wet stigmas. Penetration of stigmatic tissues is facilitated by the action of cell-wall degrading enzymes present on pollen or pollen tube walls. In grasses the group I of allergens have expansin activity and thus are thought to facilitate invasion of the pollen tube into the maternal tissues by loosening the cell walls of the stigma and style (Cosgrove *et al.*, 1997). Other enzymes such as esterases, pectinases and polygalacturonases may play an important role on breaking or hydrolyzing the stigmatic cell wall (Knox and Heslop-Harrison, 1970; Hiscock *et al.*, 1994; Mu *et al.*, 1994b; Wu *et al.*, 1996; Edlund *et al.*, 2004). A pollen specific polygalacturonase in *Brassica* was detected on the pollen tube tips during penetration of papilla cell walls (Dearnaley and Daggard, 2001). An abundant secreted peptide in lily, SCA (stigma/style cysteine adhesin), which shows some identity with LTP (Lipid Transfer Protein), has a chemotropic effect in the stigma. Moreover, a small cell wall protein, chemocyanin, which also has chemotropic effect on pollen tubes, is potentiated when combined with SCA in an *in vitro* assay (Kim *et al.*, 2003; Lord, 2003; Park and Lord, 2003).

Pollen tube growth on the transmitting tissue

The styles are the structure that establishes the connection of stigma to the ovary. Styles can be classified in three different types according to the structure of the transmitting tissue (TT): they may be open (hollow) styles, solid or semisolid (Knox, 1984; Gasser and Robinson-Beers, 1993). Rows of cells of thick walls connected by plasmodesmata and the pectin-rich middle lamellae constitute the transmitting tissue, which forms a central core in the solid styles, through which the pollen tubes grow trough the cell walls (Figs. 3C, 4 A-L, 4 J,K). In the semi-solid style an extracellular matrix (ECM) fills the spaces between the TT cells. In this case the pollen tubes grow along this ECM. In the hollow style one or more layers of glandular cells are lined along the canal of the style. These secretory cells are responsible for the components present on the stylar transmitting tissue, which is known to contain sugars, glycoproteins, proteoglycans, phenolic compounds, aminoacids and proteins (Knox, 1984; Cheung, 1996a; Wheeler *et al.*, 2001).

The components present in the ECM are thought to provide nutrients to pollen tubes, guidance, adhesion and defense against invading pathogens (Clarke and Knox, 1979; Sanders and Lord, 1992; Cheung *et al.*, 1995; Cheung, 1996a). The nutritive role of the TT is primarily supported by the divergence of pollen tube growth rates achieved *in vitro*, compared with those *in vivo*. In addition, several experiments demonstrated the incorporation of TT components in the pollen tube or depletion of reserves within the transmitting tissue by the growing pollen tubes (Herrero and Dickinson, 1979; de Graaf *et al.*, 2003).

Hydroxyproline-rich glycoproteins (HRGP) isolated from *Nicotiana* are thought to be involved in the nutrition of pollen tubes along the transmitting tissue. The *Nicotiana* transmitting tissue-specific (TTS) proteins belong to the arabinogalactan (AGP) protein family. TTS adhere to the pollen tube surface and tips, promoting pollen tube growth *in vitro* and *in vivo*. It was also demonstrated that TTS is incorporated into pollen tube walls and deglycosylated by pollen tubes. Within the transmitting tissue, TTS proteins display a gradient of increasing glycosylation from the stigmatic end to the ovarian end of the style, coincident with the direction of pollen tube growth (Cheung *et al.*, 1995; Wu *et al.*, 1995). The *Nicotiana alata* homologue was also shown to stimulate pollen tube growth *in vitro* and to attract pollen tubes in a semi-*in vitro* system (Wu *et al.*, 2000).

Other glycoproteins show differences in localization, biochemical properties and physiological activity from those described for the TTS proteins in tobacco. The Class III pistil-specific extensin-like proteins (PELPIII) are chimeric HRGP with properties of both extensins and AGPs. PELPIII is localized in the ECM of tobacco stylar transmitting tissue and was shown to be specifically directed into the callose inner wall of the pollen tubes after pollination (Bosch *et al.*, 2001; Bosch *et al.*, 2003; de Graaf *et al.*, 2004). The Galactose-rich Style Protein (GaRSPG) does not promote pollen tube growth and is localized in the cell walls of the TT (Sommer-Knudsen *et al.*, 1998). Thus, the

biological function of these proteins seems not to be directly related to pollen tube growth, but they may function as structural proteins in the pollen tube walls. Unfortunately, knock-outs of many of these style specific genes and proteins yielded no phenotype and thus their real role remains open to discussion.

The gametophytic self-incompatibility (GSI) system is controlled by a polymorphic S-locus, in which the outcome of pollination is determined by the haploid pollen genotype. This system has been characterized in a number of families, such as *Solanaceae*, *Rosaceae* and *Papaveraceae* (Wheeler *et al.*, 2001; Thomas *et al.*, 2003; Kao and Tsukamoto, 2004). The timing of rejection of pollen tubes on the style has been associated with nutrition requirements corresponding to the switch from an autotrophic growth phase to an heterotrophic growth (Herrero and Hormaza, 1996).

In *Solanaceae* and *Papaveraceae*, a S-RNase and a S-gene represent the female and male components, respectively. In the Solanaceae the S-RNase gene, which accumulates on the style, is translocated to the pollen tube cytoplasm, where it triggers a mechanism of RNA degradation in self-tubes (Luu *et al.*, 2000). Several F-box genes isolated in different species, SLF (S locus F-box)/SFB (S haplotype-specific F-box protein), found to be linked to the S-RNase gene and known to be involved in ubiquitin-mediated protein degradation, are the best candidates to confer male specificity in this cell-cell recognition system (Lai *et al.*, 2002; Entani *et al.*, 2003; Ushijima *et al.*, 2003; Qiao *et al.*, 2004, Sijacic *et al.*, 2004). Current models propose that the products of pollen S-alleles are either membrane or cell wall-bound receptors that act as gatekeepers allowing only the self S-RNase to enter the pollen tube, or otherwise the cytosolic pollen S-gene product has inhibitory properties, recognizing and inhibiting all S-RNases except those of the same allelic specificity (Thompson and Kirch, 1992; Kao and McCubbin, 1996; Luu *et al.*, 2001). Interestingly, it was shown that S-RNases can bind to several pistil proteins *in vitro*, namely TTS, PELPIII glycoprotein, the 120 KD protein, identi-

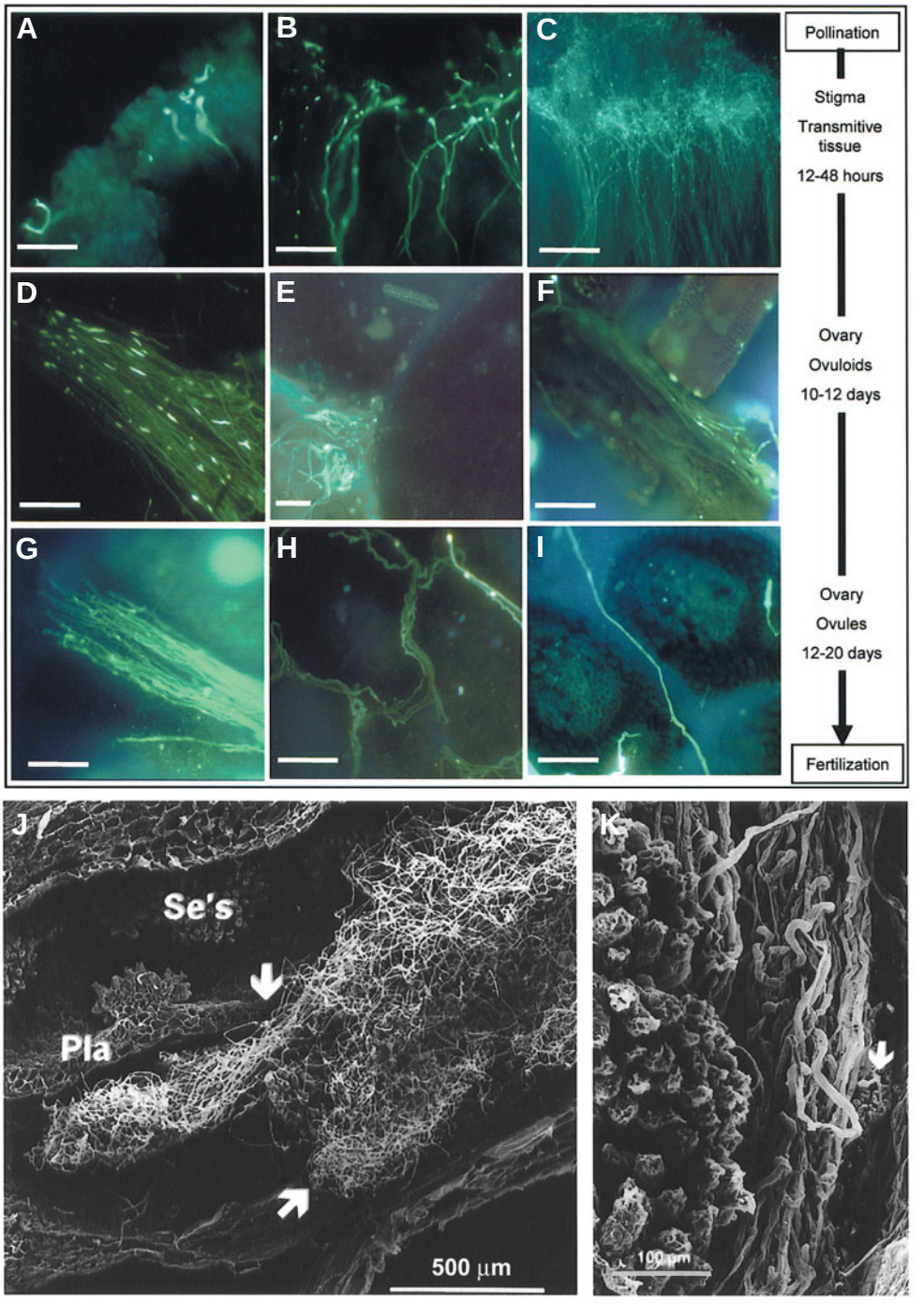


Fig. 4. Pollen tube guidance on the pistil- II. (A-I) *Pollen tube progression within the pistil of* Eucalyptus globulus. *During the first 12 hours after pollination pollen grains germinate in the papillar cells of the stigma* **(A)** *and after 24 hours the pollen tubes are growing on the transmitting tissue* **(B)**. *At 48 hours the pollen tubes are on the upper half of the style* **(C)** *and continue growing on the style until 6d. After passing the obturator they penetrate the ovary after 10 days, where they grow on the upper zone of the placenta through a zone of aborted ovules (ovuloids)* **(D-F)**. *Only after 12 days they enter the ovular zone of the placenta* **(G)** *and grow in the placenta surface around the ovules* **(H)** *until targeting the funiculus* **(I)** *and consummating fertilization. (Scale bars = 500 µm).* ***j-k.*** *Scanning electron micrographs of pollen tubes of the orchid* Ophrys lutea. *(adapted from Feijó, 1995).* **(J)** *Thousands of pollen tubes progress on the short style without any apparent order, but as soon as the transmitting tissue splits along the sides of the placenta (Pla) tubes divide into discrete 6 threadlike bundles, one on each side of the 3 placentas (arrows).* **(K)** *These bundles go along the entire placenta, but tubes "escape" at points to diverge and penetrate the ovule zone (arrow).*

fied in *N. alata* and a 11kD protein similar to a class of copper binding proteins called phytocyanins. One hypothesis is that S-RNase forms a complex with these proteins *in vivo*, facilitating the up-take S-RNases into pollen tubes (Cruz-Garcia *et al.*, 2003).

Unlike *Solanaceae*, GSI in *Papaver rhoeas* does not involve the action of S-RNases and thus S-proteins encoded by the stigma component of the S-locus interact with the pollen S-gene product, this is thought to be a plasma membrane receptor, triggering a Ca^{2+}-dependent signaling network resulting in the inhibition of pollen-tube growth. An interesting signal transduction mechanism cascade has been demonstrated to involve several components such as calcium, phosphoinositides, protein kinases, phosphatases, MAPKs and the fragmentation of F-actin during a process of programmed cell death (PCD)(Franklin-Tong *et al.*, 1991; Snowman *et al.*, 2000; Franklin-Tong *et al.*, 2002; Franklin-Tong and Franklin, 2003).

The role of the transmitting tissue on pollen tube guidance is still contentious. Two hypotheses were proposed; one relies on chemical cues, the other on the constraints exerted by the physical structure of the TT on pollen tube growth. Evidence for a mechanical stylar guidance was provided by observations that pollen grains placed on longitudinal slits within styles produced pollen tubes that were likely to grow in the direction of the stigma or towards the ovule (Mulcahy and Mulcahy, 1987). Moreover, inverted grafts of styles in lily suggested that pollen tube growth does not follow any polarized chemical gradient (Heslop-Harrison, 1987). However, several stylar proteins are able to promote growth, attract pollen tubes in a semi-*in vivo* system and establish gradients on the pistils (Mollet *et al.*, 2000; Park *et al.*, 2000; Kim *et al.*, 2003; Lord, 2003; Palanivelu *et al.*, 2003). The SCA (Stigma/stylar Cysteine-rich Adhesin) protein isolated from the style of lily combined with another small protein, a low-esterified pectin, was shown to have adhesion properties and stimulate and guide pollen tubes on an artificial adhesion matrix assay (Jauh *et al.*, 1997; Mollet *et al.*, 2000; Park *et al.*, 2000; Park and Lord, 2003). This suggests that this peptide plays a dual role in lily pollination: chemotactic in the stigma and haptotactic (adhesion mediated) in the style.

Recently a role for gamma-amino butyric acid (GABA) in pollen tube growth and guidance was proposed. The *Arabidopsis POP2* (*pollen pistil interaction2*) gene encodes a transaminase that degrades GABA and contributes to the formation of an increasing gradient from stigma to the mycropyle. *In vitro* GABA was shown to stimulate pollen tube growth, although it had an inhibitory effect at high concentrations. *pop2* pollen tubes fail to grow on the pistil, because probably they are not able to degrade the excess of GABA, while wild-type pollen tubes can grow in high concentrations (Palanivelu *et al.*, 2003). However, GABA does not have a chemotropic effect on pollen tubes *in vivo*, suggesting that other molecules may be involved in this process. Moreover, no GABA-like receptors were identified in the *Arabidopsis* genome. Therefore, further dissection of the molecular intervenients in this signaling pathway is needed.

Pollen tube guidance and targeting to the ovule

Once a pollen tube reaches the ovary, it grows along the placental tissue to an individual ovule, reorients to the funiculus and then turns to the mycropyle to reach the embryo sac (Figs. 3 A,B, D-K; 4 I-K). For some time pollen tube guidance was studied mostly by testing chemotropic attraction in *in vitro* assays. Pollen tubes were challenged to parts of the pistil placed at different distances, to homogenates of pistils or to a large range of molecules, but these experiments led to disappointing and inconclusive results in isolating an active substance in the majority of the cases (Mascarenhas, 1975; Heslop-Harrison and Heslop-Harrison, 1986; Heslop-Harrison, 1987; Reger *et al.*, 1992).

Analysis of pollen tube guidance in several ovule mutants in *Arabidopsis* indicated that the transmitting tissue should not be considered as a unique factor for pollen tube guidance and pointed at the involvement of an ovule-derived long-range activity that would account for the emergence of the pollen tubes on the surface of the septum and guidance towards the ovules over distances of about 100 μm (Hulskamp *et al.*, 1995b). However, the results were not conclusive on whether sporophytic cells of the ovule or the embryo sac itself were the source of such signals. The analysis of gametophytic mutants lacking a functional embryo sac clearly demonstrated the role of the female gametophyte in pollen tube guidance to the ovules. These results led some authors to suggest a model, in which the embryo sac cells are the source of signals that would be responsible directly or indirectly for the diffusion of chemotatic signals that would propagate through the stalk of the funiculus (Ray *et al.*, 1997).

Analysis of *magatama* (*maa*) mutants, where the female gametophyte development is delayed, showed two different stages on pollen tube guidance to the ovules. The developmental stage of *maa* female gametophytes allows pollen tube guidance from the placenta to the funiculus (funicular guidance) but is not sufficiently mature to guide them from funiculus to the mycropyle (micropylar guidance) (Shimizu and Okada, 2000). Supporting these observations is the funicular guidance impairment in the *Arabidopsis ino* mutants, in which the outer integument does not develop, but the female gametophyte is normal (Baker *et al.*, 1997). The funiculus guidance would work over a long distance from the transmitting tissue to the end of the funiculus (50-80 μm), whereas a micropylar guidance signal would be required over a short distance (Fig. 3 A-III). The failure of one pollen tube to enter the mycropyle is usually followed by the approach of another pollen tube, suggesting that the neutralization of the attraction signal would reside in one of these steps to prevent polyspermy.

In order to further study pollen tube guidance, a set of elegant experiments was designed around a semi-*in vivo* system using *Torenia fournieri,* which showed that the final attraction step of pollen tubes to the ovule resides on the female gametophyte. The naked female gametophyte protrudes from the mycropyle, making the observation of the pollen tube entering in the embryo sac possible. In this unique system pollen tubes grow through a pollinated pistil to a growth medium and were shown to be attracted to the excised *Torenia* ovules (Higashiyama *et al.*, 1998). Cell ablation experiments on ovules were successfully used to show that the two synergids on the embryo sac were the source of the diffusible attraction signal, effective at a distance of 100-200 μm. Disruption of the central cell, egg cell and integuments did not have any effect on the ability to attract the pollen tubes and a single synergid was sufficient to generate the attraction signal (Higashiyama *et al.*, 2001). An interesting observation in this system was the prerequisite of pollen tube growth through the female tissues to acquire “competence” to respond to guidance cues. These results suggest that interactions with the female tissues may modify the physiology of pollen tubes.

It is still unknown whether the guidance signal emanating from the

synergids may act directly as diffusible factor on pollen tube attraction or may also act indirectly contributing to the funicular guidance by changing the properties of the ECM of sporophytic cells (Lord and Sanders, 1992; Lush, 1999; Higashiyama *et al.*, 2003). A theory supporting a surface gradient established by a diffusible factor at distances of 100 μm can only be reasonable, if a small signaling molecule of less than 1kDa would be involved. Electric, ionic or gaseous gradients are plausible candidates for a long range activity (Crick, 1970; Ray *et al.*, 1997; Prado *et al.*, 2004).

Although the chemical that acts as a directional signal for pollen tube growth is presently unknown, the species specificity of the signal is not consistent with the classical hypothesis that calcium ions derived from the synergid act as attractants (Huang and Russell, 1992; Mascarenhas, 1993; Malhó and Trewavas, 1996; Tian *et al.*, 2000). Nitric oxide (NO), a small and highly diffusible gaseous molecule, fulfils several of the chemical requirements of a signal molecule and has been implicated in a number of developmental processes in animals and plants (Lamattina *et al.*, 2003). These features led us to investigate this molecule as an inducer of pollen tube re-orientation (Prado *et al.*, 2004). NO was shown to be endogenously produced on peroxisomes by pollen tubes, but absent from the pollen tube tip. Challenging of pollen tubes with an external NO source caused a transient growth arrest, followed by re-orientation. Downstream events on this signaling pathway seem to involve cGMP. Pollen tube negative tropism and growth is regulated by changes in NO levels at the tip of the pollen tube. An NO source could thus be present on the sporophytic female tissues, either localized on the basis of the funiculus to direct pollen tubes to the mycropyle or in sporophytic cells near the embryo sac to prevent polyspermy after fertilization. Evidence *in vivo* is still lacking, but the indication that an *AtNOS1* mutant shows fertility defects (Guo *et al.*, 2003) looks promising for a role of NO in pollen tube guidance or female fertility (Feijó *et al.*, 2004). The emerging role for a set of small peptides on plant signaling, some of them with chemotropic properties make them likely candidates, which may provide the specificity required for this mechanism (Chen *et al.*, 2000; Matsubayashi *et al.*, 2001; Lindsey *et al.*, 2002; Kim *et al.*, 2003; Wisniewska *et al.*, 2003). In maize, the *EGG APPARATUS 1* was just recently described and specifically fulfills these criteria (Marton *et al.*, 2005).

Double fertilization

The improvement of light microscopy in the 19th century allowed S. Nawashin and L. Guinard to describe two independent fertilization events in the embryo sac, one involving the union of a sperm with the central cell, leading to the endosperm and another involving the union of a sperm cell with the egg cell, which gives rise to the embryo. The origin of double fertilization is thought to have evolved from an ancestral condition in angiosperms, in which more than one nucleus was fertilized in the female gametophyte. An evolutionary genetic benefit arose from a second nutritive fertilization leading to endosperm development and assuring the success of the sibling embryo development (Friedman, 1995; Friedman and Williams, 2004).

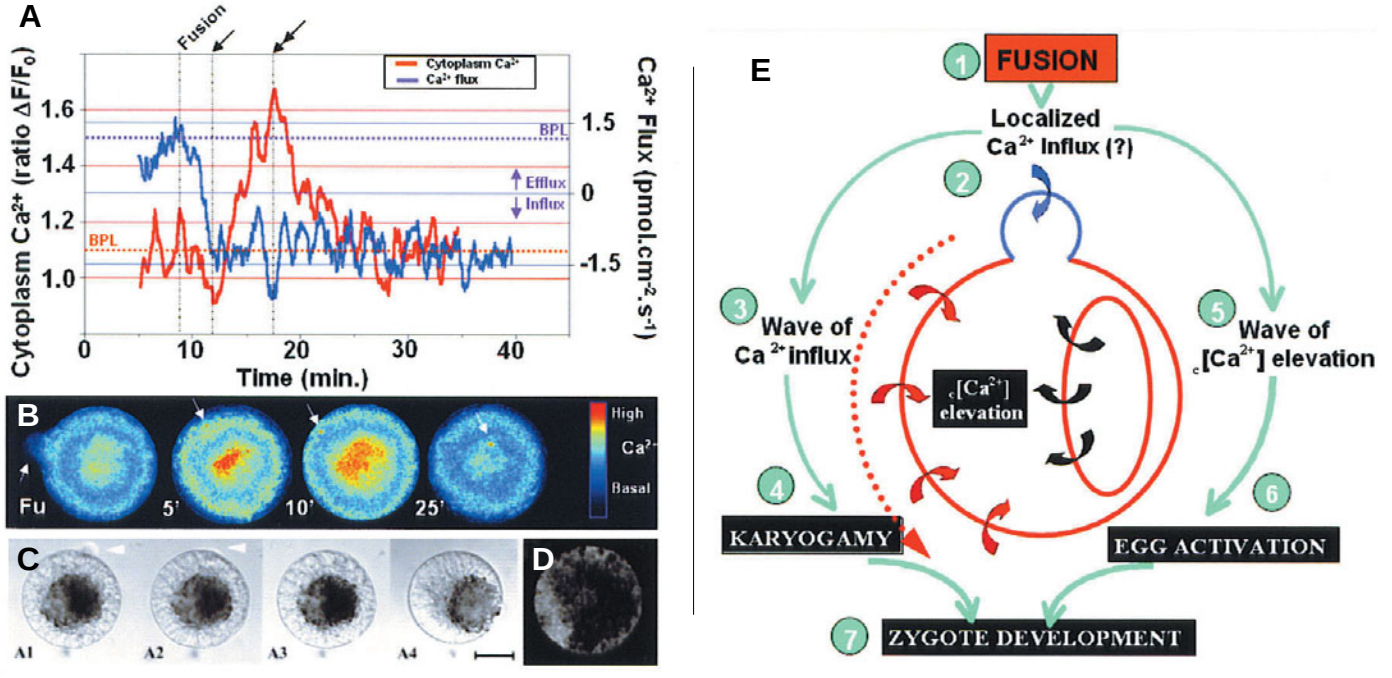


Fig. 5. Ion dynamics during egg activation. (A,B) *Simultaneous Ca^{2+} flux and* c *$[Ca^{2+}]$ measurements during maize* in vitro *fertilization. A typical experiment is shown. Time zero is set at the time when gamete adhesion is performed. Fusion is followed by the onset of a Ca^{2+} influx ((A), average Ca^{2+} influx of –1.19± 0.01 pmol.cm^{-2}.sec^{-1}) and by a transient increase of* c *$[Ca^{2+}]$* **(B)**. **(C)** *shows the sequence of events as seen by DIC and* **(D)** *a control with two-photon microscopy for the loading of the dye.* **(E)** *Diagrammatic model proposed for maize* in vitro *fertilization. Fusion (1) takes place and would trigger a localized influx of Ca^{2+} (2). Immediately after fusion a wavefront of Ca^{2+} influx is triggered (3) and after 1-3 min. cytosolic Ca^{2+} also increases (5). The influx path seems to signal mechanisms for sperm entry and possibly karyokinesis (4). Cytosolic Ca^{2+} was found to be necessary and sufficient to signal the complex set of reactions known as "Egg or Zygote activation" (6). Both paths seem to act synergistically for further egg development (7). Adapted from Antoine* et al. *(2001).*

Considerable progress was achieved in recent years on the understanding of the fundamental mechanism behind double fertilization, especially through the development of *in vitro* fertilization techniques and the use of new model species (Kranz and Lorz, 1993; Higashiyama *et al.*, 1997; Antoine *et al.*, 2000). Furthermore, the major ultrastructural events occurring during the fertilization process in *Arabidopsis* were described (Faure *et al.*, 2002).

A pre-requisite for successful fertilization is the synchronization of receptivity in all the organs and cells involved in the process. A range of signals induced by pollination are described in several species that induce physiological changes on the pistil and are related with the maturation of female gametophyte and pistil tissues (O'Neill *et al.*, 1993, Zhang and O'Neill, 1993, Mol *et al.*, 2000).

After reaching the embryo sac, the pollen tubes enter the filiform apparatus into the receptive synergid, growth arrests, the tip of the pollen tube ruptures to release the sperm cells and the male germ unit dissociates (Higashiyama *et al.*, 2000). In *Arabidopsis* pollen tube discharge was shown to occur 2-3 minutes after entry in the synergid (Rotman *et al.*, 2003). The receptive synergid undergoes cell death and this process may occur either before or after pollen tube arrival. Two aggregates of actin filaments, named "coronas" become organized during synergid cell death, one extending to the vicinity of the egg nucleus and the other occurring in the interface of the egg cell and the central cell extending to the polar nuclei. These actin aggregates are thought to be involved in pollen tube reception and help sperm cells to move and bind to their targets (Huang and Russell, 1992; Huang *et al.*, 1993; Huang and Russell, 1994). In the *Arabidopsis* female gametophytic mutant *gf2* (*gametophytic factor2*), known to encode a DnaJ chaperonin localized in the mitochondria, the synergids fail to degenerate, but embryo sacs are able to attract pollen tubes, indicating that synergid degeneration in *Arabidopsis* is not an absolute requirement for pollen tube attraction (Christensen *et al.*, 2002).

Pollen tube discharge and the consequent release of sperm cells seem to be controlled by the female gametophyte. In *feronia* (*fer*) and *sirene* (*sir*) mutants, pollen tubes keep growing throughout the embryo sac after entering it, occupying part of the central cell space and failing to discharge the sperm cells (Huck *et al.*, 2003, Rotman *et al.*, 2003). In *feronia,* synergid degeneration fails to occur, as demonstrated by normal expression of synergid-specific GUS marker lines. Apparently, no synergid rupture happens after pollen tube penetration, suggesting that a molecule present on the synergid is directly or indirectly responsible for male gamete delivery (Huck *et al.*, 2003). Morever, the embryo sacs of these mutants attract more than one pollen tube indicating that interactions between gametophytes prevent in some way attraction of additional pollen tubes.

Male and female gametophytes must also be synchronized with respect to the cell cycle to result in a successful genetic fusion product, which seems essential for the transition of the zygote to mitosis (Friedman, 1999). After pollen tube discharge the cytoplasmic content is spread inside the degenerated synergid occupying the intercellular space between the central cell and the egg cell, where gamete fusion (plasmogamy) is thought to occur (Higashiyama *et al.*, 2000; Rotman *et al.*, 2003). One sperm cell fuses with the egg cell and the other with the central cell, giving rise to the embryo and endosperm, respectively (Russell, 1992).

The development of an *in vitro* fertilization system was extremely useful to reveal the cytological and physiological events of sperm-egg cell and sperm-central cell fusion in maize (Kranz and Lorz, 1993; Faure *et al.*, 1994). The combined use of a calcium-selective vibrating probe with advanced imaging techniques demonstrated that gamete fusion triggered a calcium influx in the vicinity of the sperm entry site, which was followed by an increase in cytosolic calcium. The calcium influx spread subsequently through the whole egg cell plasma membrane as a slow wave front, which was shown to be correlated with cytological modifications induced by fertilization, such as egg cell contraction, cytoplasmic calcium elevation and cell wall assembly (Antoine *et al.*, 2000). When calcium influx was inhibited, the sperm cell fused, but was not incorporated in the egg cell. An intracellular calcium increase still occurs, presumably due to calcium release from internal stores and egg activation proceeds normally, suggesting that calcium influx is thus required for sperm incorporation and karyogamy (Fig. 5)(Antoine *et al.*, 2001).

In some species pollen mitosis II is asymmetric and the generative cell divides to produce two sperm cells with different cytoplasmic features visible at the nuclear or cytoplasm content level. It was proposed that this sperm cell dimorphism may be predetermined, giving rise to the concept of preferential fertilization (Russell, 1985; Knox *et al.*, 1993; Faure *et al.*, 2003). This would imply that some kind of pre-determination exists, in which sperm cells fuse with the egg and the diploid central cell nuclei, determining ultimately two different cell fates, the embryo and the endosperm. Under this view it is reasonable to speculate that they are endowed with some kind of molecular specificity that presently is not known (Raghavan, 2003; McCormick, 2004).

Some species seem to have evolved an alternative reproductive mechanism, apomixis, in which seed develops without any sort of fertilization. Apomixis is characterized by two important events: (1) the formation of an unreduced female gametophyte (apomeiosis) and (2) independent embryo development (parthenogenesis) that leads to the development of seeds enclosing a fertilization-independent embryo and in some cases to autonomous endosperm development. Several mutations in *Arabidopsis* have provided insights in some of the genes leading to apomitic seeds. The *fertilization-independent seed2* (*fis2*), *fertilization-independent endosperm* (*fie*) and *medea* (*mea*) mutants initiate seed development with generation of a free-nuclear endosperm, seed coat development and partial embryogenesis in the absence of fertilization, displaying a maternal-seed abortion phenotype (Ohad *et al.*, 1996; Chaudhury *et al.*, 1997; Grossniklaus *et al.,* 1998; Luo *et al.*, 1999; Ohad *et al.*, 1999; Yadegari *et al.*, 2000). *MEA* and *FIE* were shown to encode proteins from the polycomb group, characterized as transcriptional gene silencers in *Drosophila melanogaster*, whereas *FIS* encodes a zinc finger protein (Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999). The products of these genes are thought to form a complex that represses the genes involved in seed development (Chaudhury *et al.*, 1997). Embryo lethality was shown to be independent of the paternal contribution and gene dosage in *mea*, suggesting that parent-of-origin effects are involved in the control of seed development (Grossniklaus *et al.*, 1998). After fertilization all the paternal genome would be transiently silenced and embryogenesis and endosperm development would be under the exclusive control of maternal genes (Vielle-Calzada *et al.*, 1999). However, an alternative theory suggests that genomic imprinting may be established not only by modification of DNA methylation but can also be controlled by maternal gametophyte-specific gene activation, dependent on a DNA glycosylase gene, *DEMETER* (*DME*) (Choi *et al.*, 2002;

Kinoshita *et al.*, 2004). These results suggest that developmental pathways leading to asexual and sexual reproduction are interconnected and thus apomixis could be considered a deregulation of the sexual process in both time and space (Koltunow and Grossniklaus, 2003).

Conclusions

Plant male and female gametophytes and tissues interact to produce a viable embryo, in processes which are mechanically simple, but are based on sophisticated cellular and molecular interactions. All these events seem to rely on unique "decisions" that arise from primary interactions through cell walls with different intrinsic properties and represent the outcome of a rigorous regulated cell-to-cell communication process that should be endowed of some specificity and have a proper genetic specification. Both gametophytes and pistil show developmental programs that prepare these tissues for their future interactions. When first released from the sporophyte, the male gametophyte is already committed to a role, which involves challenging with cues and signaling molecules towards which it should be competent to respond. Yet, besides this generalistic pre-programming, easily evoked by *in vitro* culture, various mechanisms that happen during interaction with the female tissues provide species and individual specificity. The fact that pollen tubes grow so fast and are so easy to cultivate, makes them specially well adapted models for cellular polarization and morphogenesis studies. The female tissues form a long and complex pathway, branded by different molecular and cellular contexts that presumably are the source of multiple signals that must correctly encode the necessary information for the pollen tube to achieve guidance and correctly target the ovule. Sperm cells are then released on the female gametophyte, where specific molecular and signal interactions constitute the final checkpoint and assure that an appropriate genetic fusion product is formed, triggering a concerted developmental pathway that supports the success of a new generation and the closing of the plant life cycle.

Acknowledgements

JAF lab is supported by FCT grants POCTI/BCI/41725/2001, POCTI/BCI/46453/2002, POCTI/BIA-BCM/60046/2004 and POCTI/BIA-BCM/61270/2004. We thank Ana Catarina Certal, Sofia Cordeiro and Nuno Moreno for Fig. 2A and Anne-Frédérique Antoine for Fig. 5E.

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