Experimental Analysis of the Fertilization Process

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INTRODUCTION

In flowering plants, double fertilization is one of the defining features of reproductive development (Raghavan, 2003). Double fertilization was first discovered in 1898 by Nawaschin. It involves a complex series of interactions between essentially three plants—the male gametophyte (MG), the female gametophyte (FG), and the sporophyte—culminating in the fusion of sexual cells and nuclei and the formation of an embryo and endosperm through separate, presumably parallel, fusion and activation steps. In this review, we focus on recent advances toward understanding the mechanisms that control maturational synchrony, interaction of the MG with the FG, delivery of the male gametes, movement of sperm cells to the female gametes, fusion of the gametes, and cytological changes before and after fertilization.

The mature MG or pollen is composed of three cells: one vegetative cell that encloses two sperm cells or male gametes. The vegetative cell coordinates the delivery of the two male gametes to the FG. After landing on the stigma of an appropriate flower, a cascade of events leads to the establishment of polarity in the MG and the formation of a pollen tube. Interactions between the sporophytic tissues of the stigma, the style, and the MG guide the tube—with the two sperm cells inside—to the ovary. Within the ovary, the tube grows toward a receptive ovule, enters the ovule through the micropyle, and delivers the sperm cells within the FG to effect double fertilization. The genetic programs that govern the development of the MG and its interactions with the sporophyte are reviewed elsewhere in this issue (Edlund et al., 2004; McCormick, 2004; Kao and Tsukamoto, 2004; Sanchez et al., 2004).

The FG, or embryo sac, constitutes the structural setting for double fertilization. The FG is embedded in several diploid cell layers of the ovule and typically consists of seven cells: three antipodal cells at the chalazal end; a large central cell—precursor of the endosperm; at the micropylar end, two synergids presumed to govern the chemical attraction and delivery mechanism; and the egg cell—precursor of the zygote. Other variants on this pattern also occur (Huang and Russell, 1992). Together, the egg and synergids form the egg apparatus. Genetic programs that control the development of the FG are reviewed elsewhere in this issue (Yadegari and Drews, 2004).

Upon arrival at and penetration into the micropyle, the pollen tube delivers its two sperm cells into one of the two synergids by entering near and sometimes penetrating the filiform apparatus of the receptive synergid and subsequently rupturing. Inside the synergid, a mixture of cytoplasm of the two former cells (i.e., the tube and synergid) forms the milieu in which the nonmotile sperm cells are conveyed to the egg and central cell, where they fuse and start the development of the zygote and the endosperm, respectively. Despite a large body of knowledge concerning the events surrounding fertilization (reviewed recently by Drews and Yadegari, 2002; Lord and Russell, 2002; Willemse and van Lammeren, 2002; Higashiyama et al., 2003; Raghavan, 2003), the cellular mechanisms that control these events are still poorly understood. However, in recent years, the rediscovery of *Torenia* as a model system, improvements in the experimental manipulation of male and female gametes, advances in microscope techniques, and new molecular and genetic approaches have provided the means to obtain deeper knowledge of the underlying control mechanisms that govern the identity and behavior of the major participants in the fertilization process.

IN VIVO STUDIES OF FERTILIZATION IN FLOWERING PLANTS

Cell Cycle and Fertilization

For fertilization to be successful, the cell cycles of the male and female gametes must be synchronized. Almost all animals undergo gamete fusion in the G_1-phase of the cell cycle (with a 1C complement of genomic DNA). Seed plants appear to be an exception in that they fuse in either G_1 or G_2 in different species. Additionally, pollen may be disseminated in one of two conditions, also depending on plant species. Pollen may be shed in a bicellular condition (containing the vegetative cell and the generative cell; the latter is an immediate precursor to the sperm cells) or in a tricellular condition (containing the vegetative cell and the two sperm cells). In each condition, male germ cells may be in G_1-, G_2-, or S-phase at dissemination (Friedman, 1999). In the case of bicellular pollen, sperm cells are formed within the pollen tube. Because gamete maturation occurs during pollen tube elongation in the style and ovary, long-distance signaling likely occurs such that the male and female gametes achieve synchrony at the time of fusion. In addition to long-distance signaling, there probably also is a signal indicating that sperm and female target cells are at the same receptive cell cycle phase. Maize (*Zea mays*) and other members of the grass family tend to fuse in G_1, but others may be disseminated in S-phase

and complete DNA synthesis in the pollen tube to fuse in G_2, in synchrony with the female target cells (Friedman, 1999). In tobacco (*Nicotiana tabacum*), bicellular pollen is shed with the generative cell possessing a 2C complement of DNA (G_2). At 8 to 10 h after pollination, the generative cell divides and the resulting sperm cell nuclei remain in a 1C condition (G_1) as they approach the ovary. Within the degenerated synergid, the sperm cells complete the S-phase and appear to fuse only when they enter G_2 (H.Q. Tian, T. Yuan, and S.D. Russell, unpublished data).

Long-Distance Cues Promoting Gamete Maturation and Synchrony

Because plant sexual reproduction is based on the interplay of cells from a variety of different sources, it seems likely that mechanisms are present to ensure synchrony in receptivity as a prelude to successful gamete delivery and fusion. A clear example of long-distance maturational signaling is provided by the orchid *Phalaenopsis*. In this species, ovules do not start to develop until the stigma is pollinated, and in the absence of pollination, the flower never develops ovules. The progamic phase takes ~80 days, and the FG is fully mature when the pollen tube arrives. Auxin and the ethylene precursor 1-aminocyclo-propane-1-carboxylic acid can partly mimic this effect, but other yet unidentified primary pollination factors are needed to induce the full postpollination syndrome (Zhang and O'Neill, 1993; O'Neill, 1997). Also, in other species with shorter progamic phases, evidence for long-distance communication between gametophytes has been observed. In a number of species, synergid receptivity and pollination are correlated (Jensen et al., 1983; Russell, 1992; Christensen et al., 1997; Tian and Russell, 1997a). For instance, in *Nicotiana*, one of the two synergids becomes receptive and starts to accumulate more loosely bound calcium. Such differences in loosely bound calcium originate between the two synergids within 1 day after their formation, but they are accelerated in pollinated flowers. In addition, Tian and Russell (1997a) found that the immature FG reaches the eight-nucleate stage at 1 day after anthesis without pollination or within 12 to 18 h with successful pollination. Interestingly, at 12 to 18 h after pollination, a 1-aminocyclopropane-1-carboxylic acid synthase expression peak is present in the lower part of the pollinated style (Weterings et al., 2002).

Acceleration of other aspects of egg and embryo sac maturation also is evident in pollinated flowers. For example, male and female gametes must be synchronized with respect to the cell cycle to result in a successful fusion product. In *Nicotiana*, the egg cell and sperm cells are formed at G_1-phase but need to progress to G_2 before fusion (H.Q. Tian, T. Yuan, and S.D. Russell, unpublished data). The onset of cell cycle progression (S-phase) in the egg cell occurs in synchrony with the male gametes if the flower is pollinated near anthesis. If pollination is withheld, S-phase in the egg cell is completed at 84 h, which is more than a 1-day delay; thus, pollination may accelerate cell cycle progression in the egg cell. In maize, maturation of the female gamete also is accelerated by several hours with the presence of pollen tubes in the style (Mól et al., 2000).

In addition, there also may be long-distance signaling with respect to the restoration of receptivity in aging flowers. If *Nicotiana* flowers are pollinated as late as 5 days after anthesis, ultrastructural changes relating to senescence may be reversed, loosely bound calcium distributions will be restored, and some seed set will occur (Tian and Russell, 1997a). The nature of the synchronizing signals exchanged between the pollen tube and the FG is not yet understood.

Short-distance signaling also seems to occur between male and female gametes, because gametic fusion is delayed in *Arabidopsis thaliana* (Friedman, 1999) and tobacco (see Cell Cycle and Fertilization above) until both reach G_2. However, such short-distance signaling is suspected to involve specific cellular epitopes, whereas long-distance signaling presumably involves a diffusible stimulus.

Pollen Tube Attraction

Preceding fertilization, the pollen tube needs to be guided from the stigma to the embryo sac embedded in the ovule. Previously, it was suggested that the pollen tube may be guided by only a single chemical cue emanating from the ovule (Mascarenhas, 1993). However, models have indicated that this distance is far too long to be governed by a single gradient and may require several consecutive cues (Lush, 1999). In the transmitting tissue and along the ovarian wall, the growth of the pollen tube appears to be governed by extracellular matrix components and arabinogalactan proteins, respectively (Lennon et al., 1998; Johnson and Preuss, 2002; Sanchez et al., 2004). Once the pollen tube has emerged within the ovary, the pollen tube diverges from the septum tract and starts growing toward the ovule. Upon reaching the ovule, the tube grows up the stalk of the funiculus, typically elongating parallel to the files of cells until it nears the micropyle, where it diverges from this path and enters the ovule through the micropyle (Figure 1A) (Hülskamp et al., 1995; Shimizu and Okada, 2000). Both sporophytic and gameto-phytic tissues seem to be involved in attracting the pollen tube from the septum to the ovary.

Recently, γ-aminobutyric acid (GABA) was found to be one of the possible attractants that is produced by sporophytic tissues. In Arabidopsis, Palanivelu et al. (2003) found a sharp increase in the concentration of GABA between the septum and the inner integument cells near the micropyle of the ovule. In the gynoecium of *pollen-pistil incompatibility2* (*pop2*) mutants of Arabidopsis, GABA levels are very high as a consequence of reduced GABA transaminase expression. However, these plants still have a steep septum-to-integument GABA gradient. Pollen tubes from *pop2* plants grow at normal speed through the stylar transmitting tract of both mutant and wild-type plants, but only in the mutant ovary do they show aberrant growth; there, they fail to adhere to the ovule and do not enter the micropyle. Wild-type pollen tubes grow normally in *pop2* ovaries (Wilhelmi and Preuss, 1996). Palanivelu et al. (2003) showed that *pop2* pollen tubes are hypersensitive to GABA and suggest that they are unable to grow toward the high-GABA sources in the *pop2* ovary. However, the pollen tube–attracting activity of GABA could not be reproduced in vitro, suggesting that, together with GABA, additional molecules are needed.

Using various gametophytic and sporophytic mutants, Hülskamp et al. (1995) and Ray et al. (1997) showed that pollen
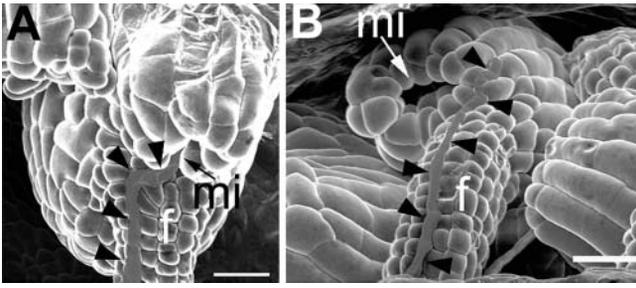
Figure 1. Scanning Electron Micrographs of Pollen Tubes Growing toward the Micropyle of Arabidopsis Ovules.

(A) Self-pollination of the wild-type ovule. The pollen tube elongates almost straight along the funiculus, then turns toward the micropyle.
(B) Self-pollination of the *magamata* FG mutant ovule. The pollen tube elongates almost precisely axially along the funiculus but loses its way near the micropyle.
Pollen tubes are indicated with arrowheads. f, funiculus; mi, micropyle. Bars = 20 μm. Adapted from Shimizu and Okada (2000) and reproduced with the permission of The Company of Biologists, Ltd.

tubes are not attracted to incompletely formed ovules or those lacking embryo sacs. These findings demonstrate that the mature, fully formed FG is necessary to guide the pollen tube from the placental tissue to the ovule, the distance of attraction not exceeding ∼200 μm. Additionally, in the recently described female gametophytic *magamata* mutants, the pollen tubes grow normally along the funiculus but lose their way when they are within 100 μm of the micropyle (Figure 1B) (Shimizu and Okada, 2000). Together, these data suggest that guidance by the FG can be subdivided into funicular and micropylar guidance.

A new opportunity to dissect the origin and nature of the FG guidance signal was provided in the model plant *Torenia fournieri*. In this plant, part of the embryo sac protrudes through the micropyle, and the egg and synergids may be manipulated directly (Erdelská, 1974; Higashiyama et al., 1997). Isolated mature ovules can be cultured in vitro and attract pollen tubes from a distance of ∼100 to 200 μm (Figure 2). Pollen tubes from *Vandellia angustifolia* do not respond to this attraction signal. Remarkably, pollen tubes can grow toward the ovules only when they have grown through the style, and this ability to capacitate the pollen tube is independent of the species of the style (Higashiyama et al., 1998, 2001, 2003; T. Higashiyama, unpublished data). Laser ablation of individual or various combinations of egg, synergid, and central cells showed that the attraction of the embryo sac is lost only when both synergids are ablated. Together, these finds show that (1) the synergids are the source of a pollen tube attraction signal, and (2) the competence of pollen tubes to respond to this directional signal requires growth in gynoecial tissues. In addition, it shows that in *Torenia* the attraction works directly via a species-distinct, diffusible signal rather than indirectly via changes induced in the extracellular matrix.

The identity of the synergid-derived attractant is not yet known. Calcium has been shown to accumulate in the synergids upon embryo sac maturation (Russell, 1996; Tian and Russell,

1997a; Kristóf et al., 1999), and pollen tubes of some species show a tropism toward calcium gradients (Reger et al., 1992a). However, this simple and general signaling molecule does not seem to be universally active in all plants. In *Torenia*, for example, the presence of 2 mM Ca^{2+} in the medium does not disturb pollen tube attraction (Higashiyama et al., 2001). Reliance upon Ca^{2+} alone as a signal does not explain why pollen tubes of different *Torenia* species fail to find ovules of other *Torenia* species (Higashiyama, 2002). Therefore, although calcium as a synergid-derived signal might attract pollen tubes, other molecules, such as sugars or peptides, also could play a role (Reger et al., 1992b; Russell et al., 1996; Palanivelu and Preuss, 2000; Willemse and van Lammeren, 2002). The role of Ca^{2+} as a second messenger in signal transduction mechanisms also may provide amplification of more specific attraction systems.

Although numerous pollen tubes elongate in the stigma and style of multiovulate flowers, the density of pollen tubes decreases as they enter the ovary and typically reaches a 1:1 ratio as pollen tubes reach the proximity of the ovule. The tendency of only one pollen tube to penetrate the ovule appears to be an important mechanism for reducing polyspermy and preventing heterofertilization (fertilization of the egg and central cells by sperm cells of different pollen tubes). The reason that only one pollen tube typically enters an ovule could be the result of (1) loss of the pollen tube attractive signal (Higashiyama et al., 2001) or (2) active pollen tube repulsion (Shimizu and Okada, 2000). Cessation of the attractive signal upon pollen tube arrival is believed to be relatively rapid. Although multiple pollen tubes elongating on the funiculus often do so on opposite sides, this fact does not resolve whether they use up an attractive or nutrient signal or produce a repulsive signal. In addition, Huck et al. (2003) found that multiple tubes can enter embryo sacs in Arabidopsis mutant *feronia* (*fer*) FGs, which argues against active pollen tube repulsion. Instead, the authors argue in favor of a rapid loss of the pollen tube attractive signal, which prevents multiple penetrations. It was also noted in *fer* FGs that the activity of other synergid genes is unchanged in the remaining synergid after
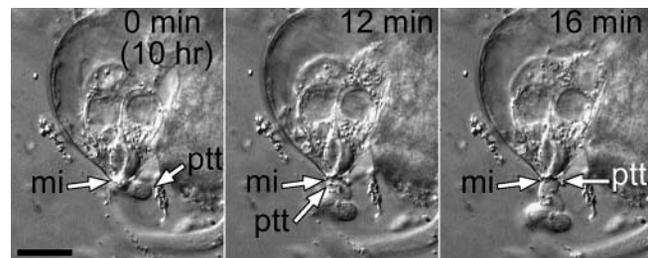
Figure 2. Attraction of the Pollen Tube to the Naked Embryo Sac of a *Torenia fournieri* Ovule in Vitro.

Images are from continuous interference contrast microscope video observations. The time after the start of video recording (from 10 h after the start of cultivation) is indicated at top right of each image. The growing tube always remains directed toward the micropylar end of the embryo sac, suggesting that the tube is attracted to this region. mi, micropylar end of the embryo sac; ptt, pollen tube tip. Bar = 30 μm. Adapted from Higashiyama et al. (2001) and reproduced with the permission of the American Association for the Advancement of Science.

pollen tube entry. Apparently, in the *fer* FG, the capacity to produce attractant is not lost immediately, which would lead to the attraction of multiple pollen tubes. More data are required to confirm this idea and to explain why multiple pollen tubes enter the same ovule so infrequently.

Although a number of pollen-expressed receptors have been identified (Muschietti et al., 1998; Kim et al., 2002), it is not yet clear which are involved in perceiving the directional cues. It is becoming clear, however, that a family of Rho small GTPases (Rop) may be involved in the ensuing change of growth direction (Yang, 2002). Studies of both pollen tube and root hair formation and growth have shown that Rop is involved in establishing and maintaining polar growth. At its localization site, Rop regulates a tip-focused cytosolic Ca^{2+} gradient, promoting and regulating the formation and dynamics of tip-localized F-actin (Li et al., 1999; Fu et al., 2001; Molendijk et al., 2001). Both are important factors for polar tip growth (Hepler et al., 2001; Moutinho et al., 2001). How Rop is localized to the future site of germination or tip growth is not yet known, but results from studies of root hair formation suggest that Rop is recruited to the future site of pollen tube germination by ADP-ribosylation-factor–GTPase-dependent, actin-independent vesicle trafficking (Molendijk et al., 2001). Together, these studies suggest that in response to directional cues from the ovule and synergid, Rop localization and activation are reoriented in the direction of this signal, changing the direction of pollen tube growth toward the micropyle (Zheng and Yang, 2000). Whether this model is true, and if it is, how the signal is perceived and processed to reorient Rop, remains to be determined. Recently, it was shown that the Actin related proteins 2 and 3 (Arp2/3) complex controls polar cell expansion of trichomes and leaf pavement cells by regulating the assembly of diffuse cortical F-actin. It is not yet clear whether Arp2/3 also controls pollen tube growth and reorientation— which are similarly dependent on cortical F-actin—because mutations in various genes that code for Arp2/3 complex proteins in Arabidopsis have not shown any effect on pollen tube growth (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a, 2003b).

Nuclear Migration and Cellular Communication in the Embryo Sac

Nuclear migration within the central cell precedes fertilization. In *Torenia* and Arabidopsis, the polar nucleus is located originally in a midchalazal position, but it moves to the center of the embryo sac and closely approaches the egg apparatus at the time of pollen tube arrival and cellular fusion (Christensen et al., 1997; Higashiyama et al., 1997; Faure et al., 2002). In unpollinated *Torenia*, the movement of the fused polar nuclei to the fertilization site also occurs but is delayed for 24 h compared with pollinated flowers. After the sperm nucleus enters the central cell, it adheres to the polar fusion nucleus, and, consistent with prior microcinematographic observations (Erdelská, 1974), the sperm nucleolus enlarges to the size of the polar nucleoli and fuses during migration. The fused nucleus migrates to a far chalazal position, where division of the primary endosperm nucleus occurs at 15 h after pollination (Higashiyama et al., 1997; Wallwork and Sedgley, 2000). By contrast, in Arabidopsis, the fused nuclei do not migrate. Soon after pollen tube arrival, the egg nucleus instead moves away from the egg–central cell boundary (Christensen et al., 1997; Faure et al., 2002).

Upon maturation and fertilization, symplastic cell-to-cell communication appears to become more restrictive. During maturation, rhodamine-labeled dextran of 10 kD passes freely between the central cell and egg apparatus cells. At fertilization, the size exclusion limit of the cell-to-cell communication is decreased to 3 kD. If pollination is withheld, the exclusion barrier also decreases to 3 kD, but this occurs 2 days after anthesis (Han et al., 2000). In either case, barriers are established that may decrease communication and therefore increase the autonomy of the fertilized egg before embryo development.

Synergid Degeneration and Gamete Delivery

Pollen tubes enter the ovule through the micropyle, traverse the nucellus (if present), and elongate within the ovule until they encounter the synergids within the embryo sac. The tube enters near the filiform apparatus, elongates into the cytoplasm of the synergid, and discharges, usually forming an aperture at the tip (Russell, 1992). In Arabidopsis, it has been determined that pollen tube discharge occurs within 2 to 3 min after entry into the synergid (Rotman et al., 2003, online video). The receptive synergid usually degenerates, and this occurs (1) before pollination, (2) as a response to pollination, or (3) upon the entry of the pollen tube (Russell, 1992, 1996). The degenerating synergid appears to assist fertilization by reducing resistance during pollen tube entry (Higashiyama et al., 2000, online video). The other, persistent synergid retains its turgor and thus may avoid entry. In addition, the degenerating synergid reorganizes its cytoskeleton to aid gamete migration (see Male Gamete Migration within the Embryo Sac below). Also, as discussed in Pollen Tube Attraction above, in a number of species the degenerating synergid accumulates Ca^{2+} (Russell, 1996; Tian and Russell, 1997a; Kristóf et al., 1999) that might act as a pollen tube attractant (Reger et al., 1992a) or might help in establishing a favorable microenvironment for gamete fusion (Antoine et al., 2000; Faure, 2001). Little is known about the mechanism that regulates synergid cell death. Some synergid-specific marker genes have been identified (Cordts et al., 2001; Huck et al., 2003), but none of them specifically labels the degenerating synergid.

In the recently described FG mutant *gametophytic factor2* in Arabidopsis, the synergids do not degenerate. The gene has been cloned and encodes a DnaJ chaperonin that is expressed throughout the plant and is localized to the mitochondria. This indicates that synergid cell death probably depends on some involvement of mitochondrial function (Christensen et al., 2002). In addition, this mutant and the *sirène* (*srn*) mutant (in which the synergids also remain intact) still are capable of attracting pollen tubes (Rotman et al., 2003), indicating that—at least in Arabidopsis—synergid cell death is not required for pollen tube attraction. In fact, experimental ablation studies suggest that only intact synergids attract pollen tubes to the ovule, underscoring the importance of both synergids in pollen tube receipt: the persistent synergid for attraction and the other, typically degenerated receptive synergid for entry of the pollen tube (Higashiyama et al., 2001).

In *Torenia* and Arabidopsis, it has been shown that soon after entry in the embryo sac, the pollen tube discharges. The pollen tube contents then flow into the space previously occupied by the receptive synergid (Higashiyama et al., 2000; Rotman et al., 2003, online videos) and thus into the narrow intercellular space located between the chalazal end of the egg cell and the micropylar end of the central cell. This area has been suggested to be the region where the sperm cells fuse with the egg and central cell (Russell, 1992).

Some conflicting data exist concerning the duration that sperm cells reside in the synergid in *Torenia*. Higashiyama et al. (1997) estimated that they are present only briefly, whereas Wallwork and Sedgley (2000) report that the sperm cells remain in the synergid for more than 1 h. For only a short period after gamete delivery are four nuclei detectable in the receptive synergid, representing the nuclei of the synergid, the vegetative cell, and the two sperm cells (Higashiyama et al., 1997). Presumably, soon after pollen tube discharge, the DNA in the exposed synergid and vegetative nuclei breaks down in the nucleases of the embryo sac (Heslop-Harrison et al., 1999).

Female Control of Fertilization

Although male and female gametes ultimately may share equally in their genetic contributions, there is accumulating evidence that the FG may regulate the delivery of the male gametes. In the Arabidopsis FG mutants *fer* and *srn*, embryo sac development is not affected and synergid differentiation and specification appear to be normal. However, after the pollen tube has entered the embryo sac, the tip of the pollen tube appears to either proliferate or grow the length of the central cell and abort, failing to discharge and deposit its sperm cells into the synergid (Figure 3) (Huck et al., 2003; Rotman et al., 2003). Similar overgrowth of the pollen tube within the embryo sac is observed after incongruous interspecific crosses in *Rhododendron* (Williams et al., 1986). Together, these data suggest that the
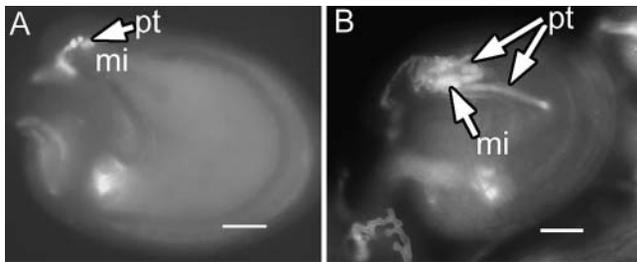
Figure 3. Female Control of Fertilization in Arabidopsis.

Fluorescence microscope images of aniline blue–labeled ovules penetrated by pollen tubes.
(A) Wild-type ovule. The pollen tube terminates in the micropylar area of the embryo sac.
(B) *feronia* ovule. The pollen tube enters the central cell after coiling in the micropylar area.
pt, pollen tube; mi, micropylar area. Bars = 30 μm. Adapted from Huck et al. (2003) and reproduced with the permission of The Company of Biologists, Ltd.

arrest of pollen tube growth and rupture are controlled directly or indirectly by a compound from the synergid. Currently, the nature of this compound is unknown, but further analysis of the *FER* and *SRN* genes, as well as other genes that disrupt FG function without otherwise affecting their development (Christensen et al., 2002; Drews and Yadegari, 2002), will help in dissecting the mechanisms that regulate gamete delivery. To date, no evidence has been found for the existence of a specific male mechanism that controls pollen tube arrest or discharge.

Gametic fusion also may be conditioned by the female. For example, in barley, the male cytoplasm is excluded from the egg, as is evident from the cytoplasmic sheath that remains within the degenerated synergid at the site of fusion with the egg. Exclusion of the sperm cytoplasm, however, does not occur during fusion with the central cell (Mogensen, 1988). Whether any inheritance of paternal plastids occurs in the offspring is determined largely by the quantity of plastids in the sperm-lineage cells, which often are excluded or eliminated progressively during development (Nagata et al., 1999). In an unusual case in alfalfa (*Medicago sativa*), there are lineages in which paternal plastids are characteristically abundant or sparse. However, the abundance of paternal plastids in the offspring of these plants is inherited only through the maternal lineage. Pollen with either sparse or abundant plastids had no predictive value in controlling the abundance of paternal plastids in the offspring. The maternal genotype predicted the outcome of the cross (Shi et al., 1991; Zhu et al., 1991).

Preferential transmission of hyperploid maize sperm cells based on B-chromosome abundance is similarly conditioned by the female (Chiavarino et al., 1998) (see Preferential Fertilization below). The ratio of B-chromosomes in the progeny is influenced strongly by the female plant. Plants displaying either high or low transmission of B-chromosomes are correlated with the maternal plant rather than the paternal plant. High or low transmission is conditioned by the maternal plant, indicating that the maternal parent controls fusion rates.

Interestingly, mate choice in plants appears to occur with similar consequences as in animals; female choice conditions the mate decisions made, whereas the male triggers this process.

Male Gamete Migration within the Embryo Sac

The complex pollen tube cytoskeletal arrays that maintain the subapical position of male gametes during tube elongation are depolymerized rapidly after pollen tube discharge, thus releasing the sperm cells from the tube, from their physical association, and from the enclosing membranes of the pollen. Presumably, this is when the cotransmission of the sperm cells ends and their developmental fates diverge. Two actin "coronas" are formed from the middle of the degenerated synergid, one terminating near the egg nucleus and the other ending near the central cell. The actin coronas, together with the myosin that is acquired on the surface of the sperm cells (Zhang et al., 1999; Zhang and Russell, 1999), appear to cooperate in the actomyosin-mediated transport of one sperm cell along the actin corona pathway to a position opposite the egg nucleus and the other sperm cell to a position near the central cell nucleus (Huang and Russell, 1994; Huang and Sheridan, 1998; Huang et al., 1999; Fu et al., 2000;

Ye et al., 2002). Cellular fusions at these sites transmit the sperm nuclei to their respective target cells.

Microinjected Alexa 488–phalloidin in the living embryo sac of *Torenia* reveals a dramatic reorganization of F-actin in the synergid and egg cells during pollen tube approach, arrival, and discharge. An actin cap located at the base of the synergid accumulates in patches along the cortex of the egg cell and develops into a linear corona track. When the sperm cells arrive within the embryo sac, they appear to follow this track to the female gametes. Soon after fusion, the actin corona disappears (Fu et al., 2000). A critical role for actin coronas during fertilization also can be concluded from a study of the fertilization of supernumerary eggs in the maize FG mutant *indeterminate gametophyte1*. Only fertilized eggs are associated with actin coronas; unfertilized supernumerary egg cells lack an actin corona (Huang and Sheridan, 1998). In addition, in the egg and central cells of nun orchids (*Phaius tankervilliae*), cellular indentations complementary to the sperm cell shape have been noted as target sites near the termination of coronas (Ye et al., 2002). Despite the important role for actin coronas in fertilization, it is not yet known how the reorganization of actin microfilaments into linear corona tracks is regulated. A template for the coronas is present before pollen tube entry (Fu et al., 2000), but most of the corona appears to assemble after the pollen tube discharges. The completion of the corona at a time when the synergid is highly degenerated suggests the incorporation of pollen tube actin. Likewise, free myosin may associate with sperm cells to form an actomyosin transport mechanism (Zhang and Russell, 1999).

Preferential Fertilization

Preferential fertilization—in which one sperm cell has a greater likelihood of fusing with the egg than the other—may theoretically occur in any of the plants in which the sperm cells differ, but it may not be visible without a specific cellular marker. The data in Table 1 suggest that sperm cells are frequently dissimilar. In *Plumbago zeylanica*, fusion between the egg and the plastid-rich sperm cell occurs in >95% of the cases examined (Russell, 1985, and subsequent unpublished observations), selectively transmitting male plastids into the zygote.

Preferential fertilization also occurs in maize, in which the sperm cell containing one extra set of B-chromosomes fuses with the egg up to 75% of the time (Roman, 1948; Carlson, 1969, 1986), but the result is interestingly variable. Rather than simply providing a marker for the sperm cell destined to fuse with the egg, B-chromosomes appear to confer a selective advantage to the sperm cell that contains them (Carlson, 1969). Not every division of the generative cell results in sperm cells with aneuploidy. In lines of maize in which B-chromosomes occur, the products of division vary, and fusion results differ (Carlson, 1969; Rusche et al., 1997, 2001). Preferential fertilization in maize is important as a populational phenomenon, but each experiment seems to result in differing degrees of sperm preferentiality (Roman, 1948; Carlson, 1969, 1986; Faure et al., 2003), and demonstrating preferentiality in individual fusions has not been possible. For example, it has not been possible to obtain preferential fertilization using the maize in vitro fertilization

system (Faure et al., 2003). Nevertheless, preferential fertilization remains an intrinsically interesting phenomenon of flowering plants that could provide considerable insight into the control of gamete maturation and identity.

IN VITRO STUDIES OF FERTILIZATION IN FLOWERING PLANTS

In the late 1980s, technical advances in isolating living, viable gametes led to the in vitro combination of male and female gametes. Initially, electrofusion (Kranz et al., 1991) and chemical induction studies (Kranz and Lörz, 1994) were introduced as reliable means of producing fusion, but a calcium-based fusion model that seems largely to mimic in vivo fertilization has attracted considerable interest (Faure et al., 1994). However, plant regeneration has not been achieved using direct products of the calcium-based fusion method. Regeneration of fertile plants and in vitro formation of endosperm have been achieved from electrofused gametes (Kranz and Lörz, 1993; Kranz et al., 1998). Electrofusions of gametes of the grasses *Coix*, *Sorghum*, *Hordeum*, and *Triticum* also have been achieved (reviewed by Kranz and Kumlehn, 1999). Attempts to combine dicot gametes chemically have met with some success in *Nicotiana tabacum* (Sun et al., 1995, 2000, 2001; Tian and Russell, 1997b), but there have been no successes at regenerating plants.

The in vitro fusion model that most closely mimics in vivo fusion uses isolated maize gametes (Faure et al., 1994) in a medium containing Ca^{2+} at a concentration of 5 mM, which appears to facilitate but not to coerce fusion. Fusion of female gametic cells is most efficient with sperm cells compared with other cells. Polyspermy is reportedly blocked successfully, and chemical signals, including a calcium influx, have been recorded (see Calcium Waves, Site of Fusion, and Egg Activation below). The model seems to work best on slightly immature egg cells (Mól et al., 2000) and is a bit technically demanding because receptivity may decline quickly after isolation. However, this model system does not seem to predict all types of gametic behavior. For example, Faure et al. (2003) reported recently that they were unable to mimic gamete preference, although in the same article they confirmed that the same gametes underwent B-chromosome–based preferential fertilization in vivo in the plant cultivar that they studied.

Some insightful in vitro fertilization experiments performed on tobacco sperm cells include comparing the fusion efficiency of somatic cells with that of a series of cells of different diameters and conducting multiple fusions. Objects of the same size class as sperm cells provided the greatest fusion efficiency among somatic cells (Sun et al., 2001). Sun et al. (1995, 2000) also tested the ability of the egg to accept multiple fusions. They found that after the first fusion, a fusion product is inhibited from fusing with another cell or organelle for a refractory period of at least 30 min. This inhibition of fusion applies to various combinations of gametes, somatic cells, and gametic-somatic combinations. Until the fused cells complete cytoplasmic reorganization, further fusion appears to be inhibited (Sun et al., 1995). This would prevent rapid serial fusions with more than one cell in the medium at a specific time. Potentially, this could inhibit

Table 1. Summary of Heteromorphism in Flowering Plant Sperm Cells Examined to Date

Pollen Type and Family	Species	Method	Morphology	Sample Size	Reference
Tricellular pollen					
Monocotyledons					
Poaceae	*Hordeum vulgaris*	3-D	I	5	Mogensen and Rusche, 1985
	Secale cereale	3-D	I	4	Mogensen and Rusche, 2000
	Zea mays	3-D	SD	1	McConchie et al., 1987a
		3-D	SD	1	Rusche, 1988
		3-D	SD	1	Rusche and Mogensen, 1988
Dicotyledons					
Carifoliaceae	*Abelia spathulata*	DAPI	OD	–	Saito et al., 2002
Brassicaceae	*Brassica campestris*	3-D	MD	5	McConchie et al., 1987b
	Brassica oleracea	3-D	MD	3	McConchie et al., 1987b
Euphorbiaceae	*Euphorbia dulcis*	3-D	MD	1	Murgia and Wilms, 1988
Asteraceae	*Gerbera jamesonii*	3-D	MD	1	Provoost et al., 1988
Plumbaginaceae	*Limonium sinuatum*	DAPI	OD	–	Saito et al., 2002
	Plumbago auriculata	DAPI	OD	–	Saito et al., 2001
	Plumbago zeylanica	3-D	MPD	11	Russell, 1984
Chenopodiaceae	*Spinacia oleracea*	3-D	MD	7	Wilms, 1986
Bicellular pollen					
Monocotyledons					
Iridaceae	*Gladiolus gandavensis*	ImAn	SD	6	Shivanna et al., 1988
Dicotyledons					
Bignoniaceae	*Campsis grandiflora*	DAPI	OD	–	Saito et al., 2002
	Tecoma capensis	DAPI	OD	–	Saito et al., 2002
Ericaceae	*Rhododendron* spp	ImAn	SD	7	Shivanna et al., 1988
Fabaceae	*Erythrina crista-galli*	DAPI	OD	–	Saito et al., 2000
	Erythrina variegata	DAPI	OD	–	Saito et al., 2002
Solanaceae	*Nicotiana tabacum*	3-D	I	9	Yu et al., 1992
	(to 30 h after pollination)	3-D	I	19	Yu and Russell, 1994
	(near pollen tube discharge)	ImAn	SD	25	Tian et al., 2001
	Petunia hybrida	3-D	I	1	Wagner and Mogensen, 1988

Methods are as follows: 3-D, serial ultrathin sectioning and three-dimensional analysis; DAPI, fluorochromatic localization on sections embedded in Technovitt; ImAn, image analysis of whole-cell profiles and/or nuclear fluorochromatic patterns. Morphology types are as follows: I, isomorphic; MD, mitochondrial cytoplasmic dimorphism; MPD, mitochondrial and plastid cytoplasmic dimorphism; OD, organelle dimorphism in which DNA nucleoids were visualized but could represent plastids, mitochondria, or both; SD, structural dimorphism (condition of cytoplasmic organellar DNA unknown). Sample size represents the number of male sperm pairs examined, unless unavailable (–).

polyspermy as well. That such a nonspecific mechanism may inhibit multiple fusions suggests a need for further studies to corroborate claims that there is a specific plant mechanism to inhibit polyspermy.

Obtaining distant hybrids and genetically modified artificial zygotes has been a frequently cited goal of in vitro fertilization, so it is interesting that transgenes in gametes and in in vitro–fertilized zygotes have now been achieved (Scholten and Kranz, 2001). The use of promoters specific to the germline (e.g., the promoter of the generative cell-specific gene *lilium generative cell 1* (*LGC1*), which is activated in the generative and sperm cells [Singh et al., 2003]) could alter gene expression in the germline to achieve novel expression in seeds or in the next generation.

Sperm Maturation

The competence of flowering plant gametes to respond appropriately to gametes of the opposite sex is acquired after gamete formation and likely involves modification of the gametic cell surface. In tobacco, for example, immediately after their formation, sperm cells placed in apposition often fuse spontaneously (Tian and Russell, 1998). In vivo, a rapidly deposited callose wall forms between the sperm cells, separating them and impeding such indiscriminate fusions (Yu and Russell, 1993). Within minutes of formation, however, sperm cells lose this tendency, and at 20 h after sperm formation, fusion can be induced only by modifying the cell surface. Exposure of the cells to dilute solutions containing both cellulase and pectinase was sufficient to trigger fusion in tobacco (Tian and Russell, 1998). Presumably, sperm maturation involves multiple carbohydrate determinants on the cell surface that reduce the likelihood of random fusions but that do not impede sexual gamete fusion. Such surface changes are anticipated to include compounds such as arabinogalactan proteins (Southworth and Kwiatkowski, 1996) and other potentially immunogenic compounds (Southworth et al., 1999). In *Plumbago*, the polarity of the male germ unit is determined by the position of the vegetative nucleus and appears to be determined in the generative cell (Russell et al., 1996).

Egg Maturation

For female gametes, the best understood model is maize, in which egg maturation is categorized into three classes (Mól et al., 2000). Small, densely cytoplasmic egg cells with a centrally positioned nucleus indicating a nonpolarized condition are classified as type A. The next stage is formed by larger egg cells that have numerous small vacuoles in the central perinuclear cytoplasm, designated type B. Mature egg cells, classified as type C, are large and highly polarized, with a prominent apical vacuole and a chalazally placed nucleus with perinuclear cytoplasm. As the silks emerge from their husks, type A egg cells are prevalent in the ovules (88%), but this decreases to nearly half (58%) at optimum silk length for pollination. Types B and C become more common near the time of fertilization.

In the brown alga *Fucus* and in many animal systems, the site of sperm entry forms the focal point of a postfertilization calcium influx, and electrical stimulation may determine zygote polarity. In animals, pricking the zygote can displace such a focal point and alter zygotic polarity. In *Fucus*, light also may reorient polarity (Goodner and Quatrano, 1993). In angiosperms, there is no such evidence of plasticity, nor is there any particular region on the female gamete that seems to be more receptive to fusion than any other region, at least according to in vitro studies (Antoine et al., 2001a). In vivo, however, position within the embryo sac appears to be an important factor in establishing both cellular identity and polarity. This polarity and orientation seem to remain unchanged during later development.

Calcium Waves, Site of Fusion, and Egg Activation

In all animal systems studied to date, gamete fusion leads to, among other events, an increase in cytoplasmic calcium ($[Ca^{2+}]_{cyt}$). This increase in $[Ca^{2+}]_{cyt}$ is believed to be essential to egg activation and occurs at the very onset of embryo development (Stricker, 1999). In maize, combining the use of a calcium-specific vibrating probe and the single-wavelength calcium fluorochrome Fluo-3, Antoine et al. (2001b) demonstrated that gamete fusion always triggers a calcium influx, which is then followed by an increase in cytoplasmic calcium (Figures 4A and 4B). The site of the calcium influx is initiated at the fusion site and within a few minutes moves in a wave-like pattern over the rest of the membrane, resulting in a homogeneous influx (Antoine et al., 2000). Thus, the initial pattern of polarity that is created at the fusion site is no longer evident after the first several minutes. In vitro, the first visible sign of egg activation is egg contraction. This seems to coincide with the increase in calcium influx and is followed by a later smoothing of the cell surface and a turgid appearance in the cytoplasm (Figure 4C). Within 20 min after fusion, the cell wall begins to form, as detected using Calcofluor white (Figure 4D).

When the Ca^{2+} channels are inhibited by Gd^{3+}, the sperm cell still can fuse with the egg cell but the sperm cell cytoplasm is not incorporated into the egg cell. Despite the blocked Ca^{2+} influx, however, the cytoplasmic calcium increase still occurs, presumably because of calcium release from intracellular stores. Together with the cytoplasmic calcium increase, egg contraction and cell wall deposition are observed in Gd^{3+}-treated cells
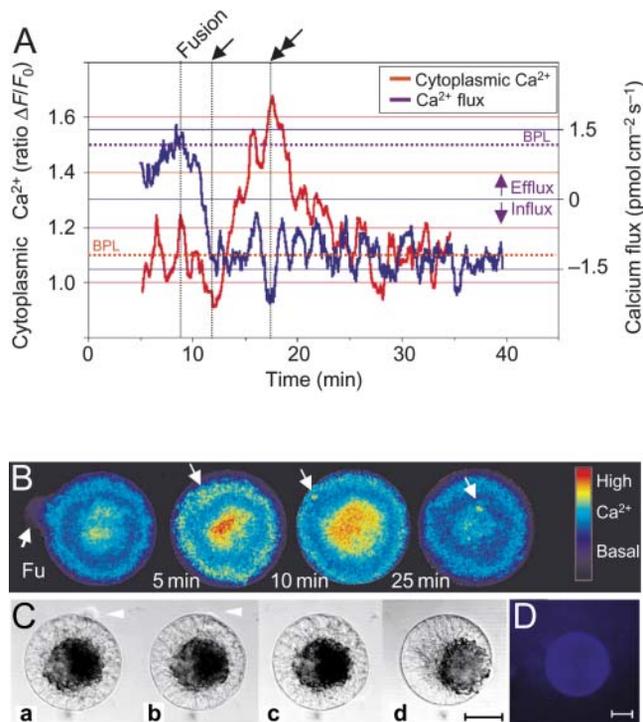
Figure 4. Calcium Flux, Cytoplasmic Calcium Levels, and Egg Activation during Maize in Vitro Fertilization.

(A) Graph representing Ca^{2+} influx (blue line) and changes in cytoplasmic Ca^{2+} levels ($[Ca^{2+}]_{cyt}$; red line). Ca^{2+} influx was measured using a calcium-specific vibrating probe, and changes in $[Ca^{2+}]_{cyt}$ were measured using the calcium fluorochrome Fluo-3 in combination with fluorescence microscopy. Time 0 was set at gamete adherence. Gamete fusion (first vertical line) is followed by the onset of a Ca^{2+} influx and by a transient increase in $[Ca^{2+}]_{cyt}$. The synchrony of the onset of the $[Ca^{2+}]_{cyt}$ increase with the stabilization of the Ca^{2+} influx (single arrow) and the synchrony of the $[Ca^{2+}]_{cyt}$ peak and the Ca^{2+} influx peak (double arrow) coincide in this experiment. BPL, basal prefertilization level. $\Delta F/F_0 = (F_t - F_0)/F_0$, where F_t is fluorescence of the egg cell or zygote as time $= t$ and F_0 is fluorescence of time $= 0$.
(B) Sequence of fluorescence microscope images from which the changes in $[Ca^{2+}]_{cyt}$ were calculated. Changes in $[Ca^{2+}]_{cyt}$ inside the egg cell are reflected by changes in the color spectrum, indicated by the vertical bar at right. Fusion (Fu) and the time after fusion are indicated at bottom left. In the first image, the adhered sperm (arrow) is visible because of Fluo-3 diffusion.
(C) Bright-field microscope images of different cytological events during maize in vitro fertilization. **(a)** Gamete adhesion (arrowhead shows the sperm). **(b)** Fusion. The sperm can be seen penetrating the egg cell. **(c)** Mild contraction of the egg cell. **(d)** Reshaping of the cell. Bar = 20 μm.
(D) Fluorescence microscope image of a 2-h zygote stained with Calcofluor white to detect cell wall deposition. Bar = 20 μm.
Adapted from Antoine et al. (2001b) and reproduced with the permission of the Nature Publishing Group.

(Antoine et al., 2001b), indicating that calcium influx is not needed to create an increased $[Ca^{2+}]_{cyt}$. The observed calcium influx instead may be needed for sperm incorporation and subsequent karyogamy. These observations allow flowering plants to be included in the general model of multicellular

organisms in which increased cytoplasmic calcium is necessary and sufficient to induce egg activation.

Han et al. (2002) studied whether the increase in cytoplasmic calcium level induced during fertilization is caused by the sperm fusion event or by a factor present in the sperm cytoplasm. They injected *T. fournieri* sperm cell extract mixed with calcium green dextran into the central cell and within minutes measured increased $[Ca^{2+}]_{cyt}$ that lasted for >40 min. Injection of maize sperm extract into *T. fournieri* did not have the same effect, indicating that the sperm cell factors may vary in different phylogenetic groups. Injection and activation of caged inositol 1,4,5-triphosphate showed that the signaling cascade triggered by the sperm factor(s) depends on inositol 1,4,5-triphosphate for calcium release. These experiments were performed in the central cell because, as was found in the maize in vitro system, it was impossible to inject the egg cell (Antoine et al., 2001b; Y. Han and B.Q. Huang, unpublished data). Although it remains to be determined whether sperm cell factors are the main cause of cytoplasmic calcium increase in the egg cell, this seems to be supported by the fact that sperm extract from flowering plants can cause calcium oscillations in mouse eggs (Li et al., 2001).

CONCLUSIONS AND PROSPECTS

Fertilization in flowering plants is a very subtle and accurate process. Compared with animals, in which hundreds of millions of male gametes may target one, often enormous, female gamete, in plants the two nonmotile male gametes target two female gametes with micrometer-level precision. Clearly, fertilization is a complex process that we are gradually beginning to understand. In recent years, we have seen advances in understanding the mechanisms that govern the interactions between the MG and FG and their gametes at cytological, physiological, biochemical, and molecular genetic levels. Using the *Torenia* system and FG mutants, we are probably very close to identifying the synergid-derived pollen tube attractants and compounds that regulate gamete delivery. Surely, studies of fertilization seem poised for more significant advances as both molecular understanding and experimental systems improve. The unique characteristics are currently being explored by genome- and transcriptome-level examination (McCormick, 2004), and powerful, high-throughput means exist to characterize the metabolic status of MG and FG cells. The incorporation of experimental methods, including microdissection, ablation, and fluorescence bleaching using lasers, in addition to increasingly precise genetic and molecular labels, represents an opportunity to understand gamete biology. Given the high number of genes found in the genomes of Arabidopsis and rice compared with mammals, including humans, it would by surprising if gametophytic biology does not occupy a significant role in the genetic complexity of plants.

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