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MINIREVIEW

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Attraction and transport of male gametes for fertilization

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Abstract Two capabilities are critical in attracting and transporting male gametes for fertilization: (1) the pollen tube must locate, enter and discharge its contents at the correct site within the female gametophyte, and (2) once inside the embryo sac, the non-motile male gametes must be transported to the egg and the central cells for double fertilization. This review summarizes current information about evidence for communication between embryo sac and pollen tube and the means by which the non-motile male gametes are transported from the aperture of the pollen tube to the site of gamete fusion.

Key words Gamete interaction · Fertilization · *Plumbago* · Pollen tube · Tobacco · Sperm cel

Introduction

Angiosperm pollen tubes each possess two highly reduced sperm cells prior to fertilization. The sperm cells are small – frequently less than 5 μm in diameter and only a fraction of the size of a typical somatic cell. Unlike non-seed plants and some of the gymnosperms, flowering plant sperm cells are non-motile and are passive participants in their movement to the female reproductive cells (review, Russell 1992). Two capabilities appear critical in attracting and transporting the male gametes for fertilization: (1) the pollen tube must locate the female reproductive cells, enter and deposit the male gametes at the correct site within the female gametophyte, and then (2) the pollen tube and the embryo sac must provide a means of transporting the male gametes to their female reproductive targets – the egg and the central cells, which are the ultimate targets of double fertilization.

This current review examines recent ideas about male gamete attraction and transportation prior to fertilization, with particular emphasis on emerging data on gametophyte-to-gametophyte interactions. Mechanistic views of pollen tube extension and their response to stimuli are outside the scope of this brief review; so, also, are long-distance signals generated by stylar interactions between pollen tubes which are reviewed elsewhere in this issue (Cheung 1996).

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Attraction and guidance of the pollen tube

True chemotropic attraction seems to be restricted to the late stages of pollen tube extension, when the tube has entered the ovary and approaches the ovules (Heslop-Harrison 1986; Mascarenhas 1993). The distinctive events of this interaction are: (1) the emergence of the tubes from the transmitting tract into the ovary, (2) their elongation along the placenta, and then (3) their divergence (frequently at 90°) to intercept and penetrate the micropyle of a selected ovule.

The origin of the ovarian signal appears to be within the ovules and appears to reside in the embryo sac itself. When specific mutant lines are used that cause the ablation of the embryo sacs but otherwise produce normal-appearing ovules, the ability to attract tubes in *Arabidopsis* ovaries is diminished, and tubes rarely penetrate ovules lacking embryo sacs. Tube emergence from the transmitting tissue is also almost eliminated (Hülskamp et al. 1995). In one particularly remarkable instance, the approach of a pollen tube was examined in a lightly pollinated flower in which the ovary contained only a single ovule with a normal embryo sac. In this situation, only one pollen tube penetrated from the transmitting tract into the ovary. Interestingly, the tube had elongated past the level of the functional ovule and entered the ovary after passing the ovule by nearly 150 μm. Upon entering the ovary, however, the pollen tube turned 180° on the placenta, growing essentially in a straight line toward the ovule, intercepting and fertilizing it (Hülskamp et al. 1995).

The above observation, in combination with numerous others from previous studies, provides some interest-

ing insights. First, there is clearly a long-distance chemotropic signal generated by the embryo sac that directs pollen tube approach from as far away as 150 μm to receptive ovules (Hülskamp et al. 1995). A further observation is that the number of tubes entering into the ovary from the transmitting tract nearly matches the number of receptive ovules in the ovary; thus, penetration of the ovary also appears to be directed by a chemotropic signal generated by the embryo sac, but whether this is the same signal as that attracting pollen tubes on the placenta is not yet clear (Hülskamp et al. 1995). Numerous studies have reported that ovules are typically penetrated by only one pollen tube and that fertilized ovules fail to attract further pollen tubes (review, Russell 1992).

Pollen tube-triggered embryo sac responses

There are also reasons to suspect that a converse pattern of signaling also exists – that elongating pollen tubes in the style appear to be able to affect signals generated by the embryo sac (Jensen et al. 1985). Synergid changes and a reversal of some features of embryo sac aging appear to be influenced by compatible pollination in some species. Whether this response is generated by the interaction of elongating pollen tubes in the style, or is generated independently by elongating pollen tubes, is not yet clear.

With respect to synergid changes, classical electron microscopic studies on fertilization have suggested that, in some species, one of the two synergids completely degenerates during tube elongation in the style. In *Gossypium*, *Hordeum*, *Linum* and *Quercus*, for example, one synergid becomes electron dense, the plasma membrane breaks down, and the contents of this synergid undergo apparent autolytic changes prior to the penetration of the pollen tube (review, Russell 1992). A remaining question has been whether these synergid changes are exacerbated by chemical fixation, which seems clearest in *Gossypium* [compare the freeze substitution results of Fisher and Jensen (1969)]. Possibly, the extreme changes evident in chemically fixed material represent an acceleration of structural events that are usually evident only after pollen tube penetration. Such fixation-sensitive changes may nonetheless represent a commitment to a developmental program that makes this synergid attractive to pollen tube penetration.

Numerous species appear to undergo none of the visible changes associated with synergid degeneration prior to pollen tube penetration, whereas, in other species, synergid degeneration may begin before pollination (e.g., *Brassica* and *Triticum*) or even before anthesis (e.g., *Beta* and *Oryza*). This variability suggests that a synergid response to the presence of pollen tubes in the style is not elicited in all flowering plants. In some species, detecting the signs of synergid receptivity may not be possible using classical ultrastructural methods (review, Russell 1992). In *Nicotiana tabacum*, subtle differences in structure exist between the two synergids prior to pollen tube penetration (Mogensen and Suthar 1979), but loss of viability and the plasma membrane, as assayed by fluorescein diacetate, does not occur until the arrival and discharge of the tube into the receptive synergid (Huang and Russell 1992). Electron opacity of the cytoplasm occurs only after the arrival and discharge of the pollen tube and this is a more gradual process in freeze-substituted material (Huang et al. 1993b). A consistent marker of the determination of the degenerate synergid in tobacco appears to be the labeling of the nucleus of the receptive synergid with calcium-induced potassium pyroantimonate precipitates between 1 and 2 days after anthesis. This early change appears to occur regardless of whether the flower is pollinated, but synergid changes appear to be accelerated in pollinated flowers (Tian and Russell, unpublished data).

A topic that has received sparse attention is the means by which the embryo sac loses its ability to attract pollen tubes through senescence. In tobacco, flowers are typically shed on the 8th day after anthesis; the egg apparatus displays degenerative changes as early as the 4th day, and by the 5th day the ovule has lost much of its normal labeling pattern of calcium-induced potassium pyroantimonate precipitates. If flowers are pollinated on the 5th or 6th day, however, prior amounts and distributions of precipitates are mostly reachieved within 2 days, and many of the ovules are fertilized, albeit in lesser numbers (Tian and Russell, unpublished data). This observation suggests that the ovule and the embryo sac are responsive to a signal provided by pollination that triggers a rejuvenation of calcium concentrations in the ovule, supporting the existence of a pollen tube generated signal.

Nature of the pollen tube chemotropic signal

The chemotropic signal that guides the pollen tube has been the subject of numerous studies (see Reger et al. 1992). A number of pollen tube attractants have been proposed, including simple inorganic ions like calcium, simple sugars like glucose, and small proteins (review, Heslop-Harrison 1986; Reger et al. 1992). Currently, the major attention is being given to the location of calcium and insoluble polysaccharides, but there is emerging evidence that arabinogalatoproteins may be present in the walls of the micropyle in some species (S. Coimbra and R. Salema, personal communication); these could also play a role in attracting pollen tubes. As modern molecular techniques and functional mutants are developed, hopefully the role of each factor may be separately considered.

Structural studies indicate that calcium is present in extremely high concentrations within synergids (see Reger et al. 1992), but these concentrations alone do not explain how a directional signal could be provided. One feature that has only recently been considered is that calcium may exist in a variety of bound and free states within the cell and the extracellular matrix; this feature

may provide responsiveness to the signaling mechanism. This directional signal is unlikely to be truly free calcium, which may diffuse too readily to maintain a signal, whereas tightly bound calcium would likely be immobile and difficult to regulate as a signal. Calcium that is more loosely bound to the matrix would more likely be available for exchange or could be converted into other solubility states to control expression. In fertilized ovules, chlorotetracycline labeling is particularly dramatic in degenerated synergids, indicating the presence of abundant membrane-bound calcium (Huang and Russell 1992). This increase in chlorotetracycline labeling suggests that either abundant calcium is imported that becomes bound to membranes in the degenerated synergid or some of the abundant calcium in the synergid is converted to this bound form. The ability to control calcium pools offers unique opportunities for control. Calcium changes, however, are not the exclusive chemotropic mechanism; in some plants, they do not appear to be significant factors (review, Heslop-Harrison 1986).

Sperm cell transport in the pollen tube

The male germ cells are ultimately dependent on the pollen tubes for their delivery to the ovule, and this is reflected in their lack of independent motility. Sperm cells and their predecessor generative cells possess abundant microtubules, but these occur in crosslinked configurations that would apparently preclude motility (Palevitz and Tiezzi 1992). Further, the cells appear to lack actin – thus constituting an exception among the types of plant cells described to date (Palevitz and Liu 1992). The cells of the male germ lineage must, therefore, be conveyed passively through the pollen tube and, further yet, into alignment with the egg and central cell prior to double fertilization.

The current model for gamete movement in angiosperm pollen tubes involves the abundant cortical bundles of F-actin that are found axially aligned in the pollen tube cytoplasm. These are believed to interact with myosin, which has been localized on the surface of the vegetative nucleus and the generative cell (Heslop-Harrison and Heslop-Harrison 1989b; Tang et al. 1989) and, later on, the sperm cells (Cresti, personal communication). According to Miller et al. (1995), this represents myosin I, whereas myosin IV and V are associated with pollen tube organelles involved with tube extension.

Throughout their descent in the pollen tube, the male germ cells appear to remain physically associated in a male germ unit (Mogensen 1992) that is transported through ATP-dependent actomyosin interactions. In addition to the structural data, the dependence of male germ unit movement on actomyosin interactions is supported by cytochalasin B inhibition, which appears to act by depolymerizing actin filaments and by inhibiting the replenishment of ATP, both of which are required for actomyosin-based movement (Heslop-Harrison and Heslop-Harrison 1989a).

The exact site of myosin on the male germ cells has been regarded as problematical; however, the most reasonable conclusion seems to be that the myosin is associated with the inner surface of the membrane of the pollen tube that surrounds the male germ cells. In support of this model, Heslop-Harrison and Heslop-Harrison (1989b) observed that anti-myosin labeling of generative cells could be removed during processing, and that the label appears to remain on a membranous sheath normally surrounding the cell in vivo, which would undoubtedly represent the inner pollen tube plasma membrane. This surrounding membrane remains intact during the passage of the male germ cells inside the tube, and myosin would thereby be suitably presented to the actin bundles to permit actomyosin-based movement during normal tube extension. Colloidal gold labeling of myosin on the male germ cells in vivo is inconclusive, though, because of the low concentration of label used in immunoelectron microscopy and the length of the antibody ligand, which exceeds the width of the membranes involved; labeling of isolated sperm cells has not produced any evidence of myosin to date (Z. Zhang and S.D. Russell, unpublished data). Since the outer membrane is lost during fertilization (review, Russell 1992), the exact location of myosin could be critical in any further transport of the sperm cells once they are deposited in the embryo sac. Elsewhere in cell biology, myosin is found strictly as an intracellular component.

Sperm cell transport within the embryo sac

Once discharged from the pollen tube, the sperm cells are freed from their association with the vegetative nucleus and lose their ensheathing vegetative cell membrane, exposing their own plasma membranes within the embryo sac (review, Russell 1994). Concomitant with this, the actin bundles present in the pollen tube disorganize and only small punctae remain fluorescent when labeled with rhodamine phalloidin.

Within normally organized embryo sacs, two prominent bands or “coronas” are present that label intensely with rhodamine phalloidin and which occupy the future pathway of the male gametes. These bands appear to be organized prior to the arrival of the pollen tube in tobacco (Huang et al. 1993b; Russell 1993; Huang and Russell 1994) and maize (Huang and Sheridan 1994), which are the only plants studied in detail to date. One of the bands forms at the chalazal end of the synergid, extending from the middle lateral region of the degenerated synergid to the region of the egg nucleus, conforming to the chalazal boundary of the synergid. The second band occurs between the egg and central cell and extends from the lateral edge of the egg cell to the region of the polar nuclei (Figs. 1, 2).

These bands consist of aggregates of material that appear electron translucent and essentially homogeneous when viewed using the transmission electron microscope. In tobacco, this region can be labeled densely us-

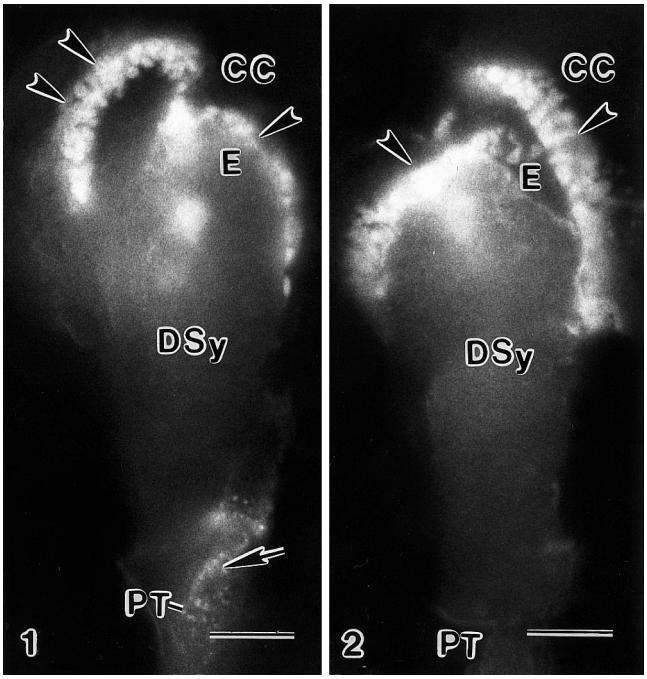

Fig. 1 Isolated embryo sac of tobacco soon after arrival of the pollen tube labeled with rhodamine-phalloidin. Brightly-labeled aggregates (*arrowheads*) form two coronas or bands of F-actin at the chalazal end of the degenerated synergid (*DSy*) and in interfaces between the egg (*E*) and central cell (*CC*). Note punctate actin labeling (*arrow*) in the terminal region of the pollen tube (*PT*). From Huang and Russell (1994), reproduced by permission. ×1170. *Bar* 10 μm

Fig. 2 Different focal plane of Fig. 1, showing actin aggregates of corona (*right arrowhead*) between the egg (*E*) and central cells (*CC*) and at the chalazal end (*left arrowhead*) of the corona near the degenerated synergid (*DSy*). *PT*, Pollen tube. From Huang and Russell (1994), reproduced by permission. ×1170. *Bar* 10 μm

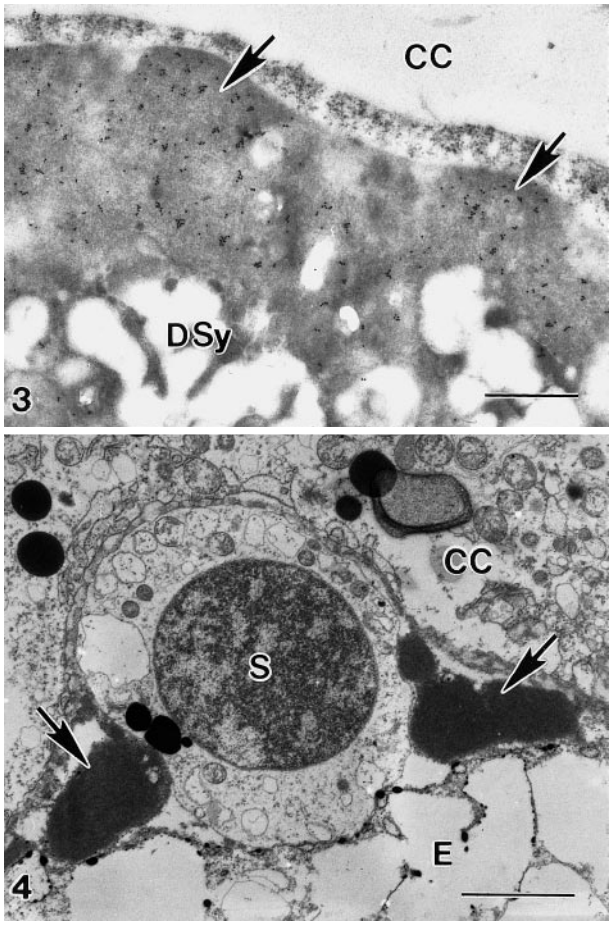

Fig. 3 Immunogold electron microscopic localization of actin in freeze-substituted ovules of tobacco. *Arrows* indicate electron-dense bodies containing anti-actin immunogold label near the chalazal end of the degenerated synergid (*DSy*), adjacent to the central cell (*CC*). From Huang and Russell (1994), reproduced by permission. ×12 500. *Bar* 1 μm

Fig. 4 An unfused sperm cell (*S*) of barley lodged between the egg (*E*) and central cell, just beyond the degenerated synergid. Osmiophilic bodies (*arrows*) seen adjacent to the sperm cell likely represent actin bands. From Mogensen (1982), reproduced by permission. ×7700. *Bar* 2 μm

ing an anti-actin monoclonal antibody in conjunction with a colloidal gold-conjugated secondary antibody (Fig. 3) (Huang and Russell 1994).

Although anti-actin localizations are not available for other species, similar bodies have been reported or illustrated in comparable locations between the egg, central cell and synergid in *Arabidopsis*, *Beta*, *Brassica*, *Capsella*, *Glycine*, *Gossypium*, *Helianthus*, *Hordeum*, *Plumbago*, *Spinacia* and *Triticale* (reviews, Russell 1992; Huang and Russell 1994). In *Hordeum* (Mogensen 1982), the relationship between one such band and the unfused male gamete has been illustrated next to the summit of the egg cell (Fig. 4). The sperm cell is located between two osmiophilic aggregates of the apparent band in this figure, in which the band appears to be sectioned longitudinally. The number of such bands appears to vary with respect to the characteristics of the fertilization system. In *Plumbago*, which lacks synergids, the number is reduced to only one such band (Huang et al. 1993a), whereas in the indeterminate gametophyte mutant of maize, in which more than one fertile egg is frequently seen, the number of such bands is usually greater than the two normally seen in wild-type maize embryo sacs (B. Huang and W. Sheridan, personal communication).

Presumably, actin bands in the embryo sac interact with the sperm cells to transport the gametes to their site of fusion. The myosin that coats them, however, is problematical since the membrane that normally envelopes them in the pollen tube is lost during their discharge. Using isolated sperm cells lacking this outer membrane, it has proven impossible to demonstrate the presence of myosin on the surface of the sperm cells. Attempts to demonstrate the presence of myosin have included: (1) mobility experiments using artificial actin matrices, (2) mobility experiments using natural actin matrices without native cytoplasm present and (3) indirect immunolabeling including electrophoretic assays for antibody label (Zhang and Russell 1995). Sperm cells do, however, track actin if they are introduced into the cytoplasm of

streaming *Nitella*, which is known to contain free myosin. Negatively charged latex beads with binding sites for protein (Polybead polystyrene 6 μm beads, Polysciences, Warrington, Pa., USA) injected into the cytoplasm of *Nitella* will also track actin, unless treated with an immunochemical block (e.g., bovine serum albumin, milk proteins), demonstrating that the beads are not simply migrating because of the force of cytoplasmic movement (Zhang and Russell, unpublished data). These observations considered together suggest that the sperm cells acquire most if not all of the myosin from the cytoplasm surrounding the discharged sperm cells. The most likely source is the cytoplasm of the pollen tube, but the synergid is another potential source. Assays using cell electrophoresis indicate that sperm cells have a negative surface charge at physiological pH (Zhang and Russell 1995).

Although an actomyosin-based transport system for the movement of sperm cells seems to be the most likely, an interesting alternative suggested by other cell biology studies is a pattern of movement similar to that described in the parasitic bacterium *Listeria monocytogenes*. *Listeria* is known to enter the cytoplasm of the host and catalyze the polymerization of host actin to propel the bacteria within the host (Theriot et al. 1992). This polymerization is apparently catalyzed by the actin-binding protein ActA, which is asymmetrically distributed on the trailing and lateral surfaces of the bacterium and which is passed on by cell fission from one generation to the next (Kocks et al. 1993).

Although the outward characteristics of *Listeria*'s host-facilitated movement appear consistent with the actin bands in flowering plants, there are important differences. The distribution of actin in *Listeria* trails is essentially tubular, possibly because G-actin available for polymerization is more plentiful on the sides of the bacterium than in its immediate wake, and distinct fibrils are evident in the trail (Tilney et al. 1992). Also, the actin coronas described in *Plumbago*, *Nicotiana* and maize appear to precede the arrival of the sperm cells. Further, the coronas are distributed in groups of actin aggregations located exclusively at the periphery of the cells of the egg apparatus, and the sperm cells do not associate with the actin bands in a polarized manner during their transient presence in the synergid. Despite the elegance of the *L. monocytogenes* model in describing movement in a host, it does not appear to apply to short-distance sperm transport in angiosperms.

Although the universality of an actomyosin-based mechanism of short-distance sperm transport is clearly unproved, actomyosin interaction provides a partial explanation for how sperm cells may be passively transported to the site of gamete fusion in the embryo sac. The means by which the patterning of actin coronas or bands occurs, and how the path of actin coronas may be changed by the arrival of the pollen tube and sperm cells are important questions that are currently being pursued.

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