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ORIGINAL ARTICLE

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Sperm movement during double fertilization of a flowering plant, *Phaius tankervilliae*

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Abstract Fertilization in flowering plants involves the fusion of one sperm with the egg cell and a second sperm with the central cell. In the Nun orchid, *Phaius tankervilliae* (Aiton) Bl., the pollen tube deposits two sperms in the "cytoplasmic mass" of the degenerating synergid. Initially both sperms stay close together. Soon afterwards, the two sperms undergo migration. The leading sperm migrates towards the central cell, while the other sperm moves laterally towards the egg cell. The degenerated synergid cytoplasmic content fills in the available space between the central cell and the egg cell, forming the actin coronas. Due to the high actin content, the bright fluorescence initially prevents the visualization of cellular details. With the subsequent reduction in fluorescence, actin staining reveals that the two sperms are pear-shaped with pointed tails. As the sperms approach their respective target cells, cellular extensions form near the point of sperm entry in both the egg cell and the central cell. These structures appear to aid in the cell fusion process. The morphological and structural features observed provide evidence that the process of double fertilization requires the active participation of not only the two sperms but also the egg cell and the central cell.

Keywords Actin corona · Central cell · Egg cell · Sperm cell · Synergid · *Phaius* (double fertilization)

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Abbreviation DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride

Introduction

Since the discovery of fertilization in the 19th century (see Batygina and Vasilyeva 2001), the events of fertilization have fascinated scientists (Russell 1992; Faure and Dumas 2001). However, the inaccessibility of ovules and the small size of the gametes have provided a challenge to biologists interested in plant reproduction. Jensen and co-workers detailed the fundamentals of the fertilization process in plants (Jensen 1974). Although the general process is clear, dynamic events related to the process of fertilization within the embryo sac remain sketchy.

One of the limitations to the study of double fertilization in flowering plants is that this event is completed within a short time. In *Torenia fournieri*, it is estimated that this process is completed within 10 h from pollination and approximately 3 h after the sperms arrive at the embryo sac (Higashiyama et al. 1997). Furthermore, it is technically difficult to capture details within the degenerated synergid as the embryo sac is enclosed by integuments in a majority of flowering plants. In this study, we make use of the unique reproductive features of orchids (Yeung and Law 1997) to further our understanding of fertilization in plants. In the Nun orchid, *Phaius tankervilliae*, we discovered that the process of fertilization from pollen tube entry into the embryo sac to the gamete union requires approximately 36 h. This window of time allows for the study of fertilization events. The other advantage of using orchid ovules is that numerous fertilized ovules are present. The ovules are small in size and the embryo sacs are covered by thin integuments, having just four layers of cells. Hence, developing embryo sacs and embryos are readily isolated after a mild digestion using cell-wall degrading enzymes (Ye et al. 1997; Tung et al. 2000). The small size of the ovules allows for better fixation and staining of the

cellular components and for improved visualization of the entire embryo sac contents using fluorescence and laser confocal microscopy. As a result, we were able to capture and document additional events that take place within the degenerating synergid at the time of fertilization.

In order to visualize changes in cells within the embryo sac, we stained for actin filaments using rhodamine-phalloidin. Actin filaments are one of the major cytoskeletal elements in plants and have many important cellular functions. The localization of actin filaments could also mark the boundary of cells and allow us to visualize changes near the cell surface. This method was chosen because labeled phalloidin penetrates into the embryo sac quickly without the need for coupling to a secondary antibody for the visualization of actin.

In this study, we document changes in the sperm cells, the egg cell, and the central cell during the fertilization process. The observations suggest that the process of double fertilization requires the active participation of not only the two sperms but also the egg cell and the central cell.

Materials and methods

Plant material

The Nun orchid, *Phaius tankervilliae* (Aiton) Bl., was maintained in the greenhouse at the University of Hong Kong. The plants flower once per year. Anthesis occurred at the end of February. Once the flowers opened, they were hand-pollinated. With successful pollination, ovaries began to swell. Fertilization occurred approximately 38 days after pollination. Ovaries were sampled continuously from late March to mid-April to ensure that we could capture as many stages of fertilization as possible. The information presented in this manuscript represents three years of studies.

Fixation and immunolabeling

Ovules were excised from ovaries and immediately pre-incubated in MFBS buffer (50 mM Pipes, 5 mM EGTA, 5 mM $MgSO_4$, 8% sucrose, pH 6.9) containing 1% dimethyl sulfoxide (DMSO), 0.5% Triton X-100, and 400 μM *m*-maleimidobenzoic acid N-hydroxysuccinimide ester for 45 min. After pre-incubation, the ovules were washed three times (10 min each) in the MFBS buffer and fixed immediately in freshly prepared 4% paraformaldehyde in MFBS buffer containing 1% DMSO and 0.5% Triton X-100 for 10 min. The ovules were then rinsed in MFBS buffer (3×) and then digested with an enzyme solution containing 1% cellulase (Sigma), 1% pectinase (Sigma), and 0.3% pectolyase (Sigma) for 5–6 min at 37 °C. The samples were then rinsed in the MFBS buffer (3×) followed by phosphate-buffered saline (PBS: 0.137 M NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.2; 3×). The embryo sacs were isolated by hand-dissection with needles using a Leica DM IRB inverted phase-contrast microscope. The isolated embryo sacs were collected in a small vial or placed directly on a poly-L-lysine-coated cover glass. The specimens were incubated in a rhodamine-phalloidin solution (Molecular Probes, Eugene, Ore., USA) diluted 1:50 with 1% bovine serum albumin in PBS and 1 mg/l 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) at 4 °C for 4–8 h. The samples were then rinsed in distilled water and mounted in a Slowfade solution (Molecular Probes) to reduce fading. Controls were processed in a similar manner except without rhodamine-phalloidin stain. Controls showed no actin fluorescence. It is important to note that we have also tested the antibody method of actin staining; however, rhodamine-phalloidin provides the best results. The one-step staining procedure using phalloidin allowed us to process a large number of ovules within a short period of time.

A BioRad MRC-600 confocal laser-scanning microscope mounted on a Nikon Optiphot-2 microscope equipped with an argon ion laser was used to examine and capture images of the rhodamine-phalloidin-stained whole-mounted embryo sacs. The corresponding position of the nuclei within the embryo sac was captured using a Leica DMR fluorescence microscope equipped with the A filter combination (UV excitation 340–380 nm, emission 425 nm).

Nuclear staining using DAPI

An additional set of embryo sacs was dissected and stained with DAPI in order to study the movement of the sperm cells during the fertilization process. Ovules were fixed overnight using 1.6% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8). After fixation, the samples were rinsed in buffer (3×) and then subjected to cell-wall digestion as indicated above. Embryo sacs were isolated and then stained using DAPI (1 mg/l in PBS). The preparations were examined and the images were captured using a Leica fluorescence microscope as indicated above. The green fluorescence was examined using the I3 filter combination (blue excitation 450–490 nm, emission 515 nm). The images were captured using the Leica Image Data Archive imaging system.

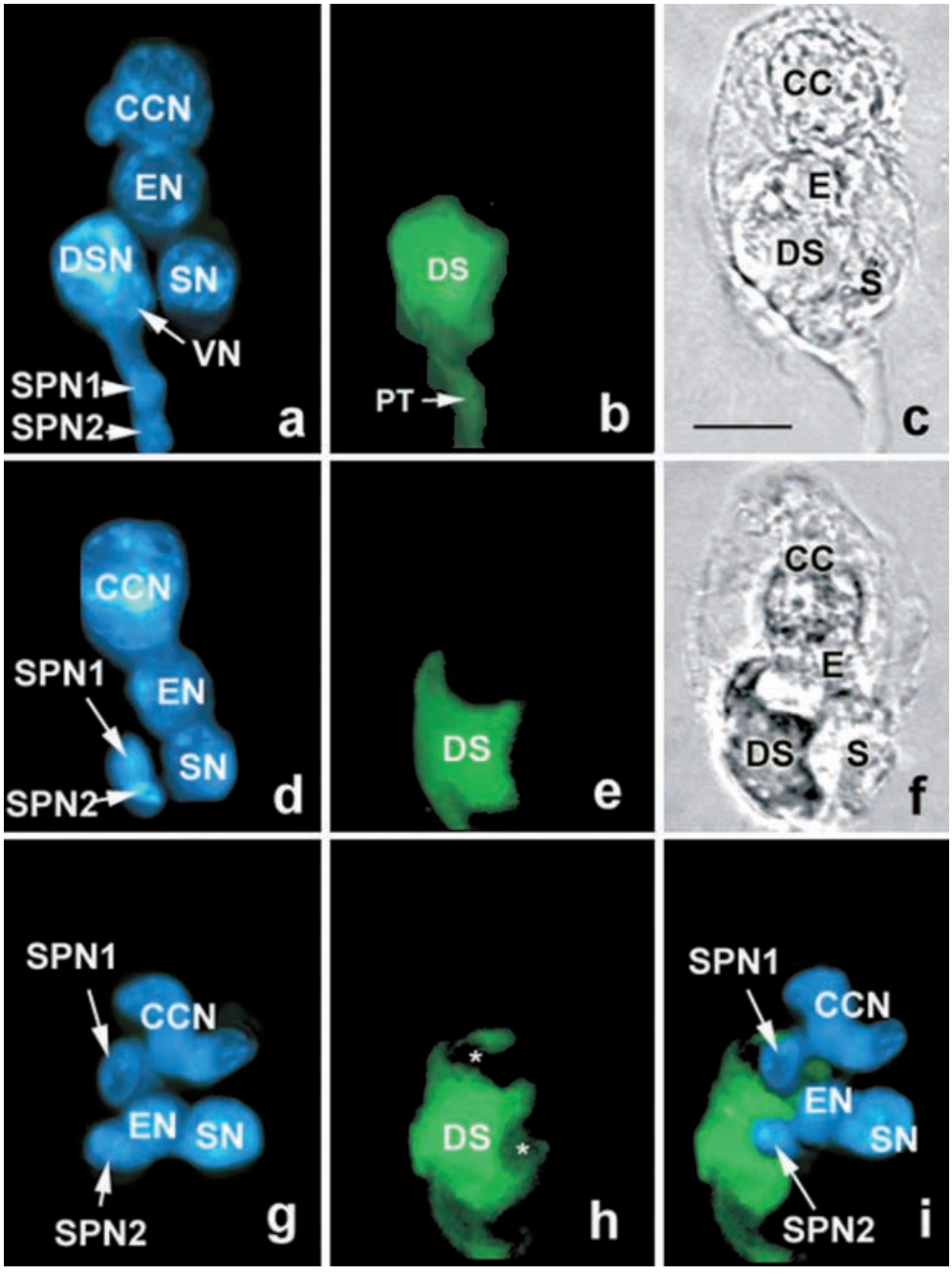
High-resolution light microscopy of plastic sections

The protocols for histological and histochemical studies were similar to those detailed by Yeung (1999). In brief, the ovules were fixed using 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05 M phosphate buffer (pH 6.8), for 24 h, dehydrated using methyl cellosolve followed by two changes of absolute ethanol, and then infiltrated and embedded in Technovit 7100 (Kulzer and Co., Wehrheim, Germany) embedding medium. Serial 3-μm-thick sections were cut with glass knives on a Reichert Autocut rotary microtome. For general histological examination, the sections were stained by the periodic acid-Schiff (PAS) procedure and counterstained with 0.05% (w/v) toluidine blue O in benzoate buffer (pH 4.4) (Yeung 1984). Histochemical staining of protein and total carbohydrates was performed according to Yeung (1984). The sections were stained with the PAS reaction for total carbohydrates, and counterstained with amido black 10B for proteins. Red coloration indicates carbohydrates and blue coloration indicates proteins.

Results

The entry of the pollen tube into the degenerating synergid

As in a majority of flowering plants, the pollen tube enters the degenerating synergid of the embryo sac, delivering the two sperms. The vegetative nucleus from the pollen tube enters first, followed by the sperms (Fig. 1a). The degenerating synergid nucleus and the vegetative nucleus of the pollen tube disintegrate rapidly and can no longer be discerned soon after the entry of the sperms (Fig. 1d). Initially, both sperms stay close to one another (Fig. 1d). The distal sperm then moves towards the central cell while the other moves principally laterally towards the egg cell (Fig. 1g, i). The fate



of the sperms is predetermined. The sperm nearest to the tip of the pollen tube is destined to fuse with the central cell and the other sperm will fuse with the egg cell.

During the course of this investigation, the autofluorescence characteristic of the isolated embryo sac and pollen tube was also studied. When the samples are examined with a fluorescence microscope equipped with the I3 filter combination, a very weak green autofluorescence is found in the degenerating synergid and the pollen tube, beginning at the time of pollen tube entry into the embryo sac. After DAPI staining the contents of the degenerated synergid fluoresce bright green, especially after pollen tube entry (Fig. 1b, e, h). The nature of this fluorescence is unclear. However, such an image enabled us to observe the flow of the degenerated synergid without the need for elaborate processing and staining. Furthermore, the location of sperm nuclei could clearly be discerned at the same time when viewed with the UV excitation filter combination. The green fluorescence image indicates that the degenerated synergid content flows into the space towards the egg cell and the central cell after sperm entry (Fig. 1e, h). Interference contrast images illustrating all cell structures, especially the nuclei, within the embryo sac, concur with the fluorescence

Fig. 1a–i The distribution of nuclei within the embryo sac of the Nun orchid (*Phaius tankervilliae*) after DAPI staining, and the fluorescence of the degenerated synergid and pollen tube viewed with the I3 filter combination during fertilization. **a** DAPI staining clearly reveals all the nuclei (CCN, EN, DSN, SN, VN, SPN1 and SPN2) within the pollen tube and the embryo sac. **b** When the same section was examined using the I3 filter combination, a green fluorescence is observed in the degenerating synergid as well as in the pollen tube. **c** Interference-contrast image of the same embryo sac as shown in **b**. **d** After entering the degenerating synergid, the sperms initially stay close to one another. The vegetative nucleus of the pollen tube and degenerating synergid nucleus have both been degraded and are no longer visible. **e** The green fluorescent material extends towards the egg cell and the central cell from the degenerating synergid. **f** The corresponding interference-contrast image also reveals that the cytoplasmic content begins to flow towards the egg cells and the central cell. **g** The sperms separate from one another and migrate towards their respective partners. **h** The green fluorescence indicates the flow of the synergid content and the corresponding locations of the sperms are indicated (*asterisks*). **i** When DAPI and green fluorescent images are superimposed, it is clear that sperms have migrated in different directions within the degenerating cytoplasm with sperm cell 1 migrating towards the central cell and eventually fusing with it to form the endosperm, and sperm cell 2 migrating laterally towards the egg cell resulting in the formation of a zygote. *CC* Central cell, *CCN* central cell nucleus, *DS* degenerating synergid, *DSN* degenerating synergid nucleus, *E* egg cell, *EN* egg nucleus, *PT* pollen tube, *S* persistent synergid, *SN* persistent synergid nucleus, *SPN1* sperm nucleus 1, *SPN2* sperm nucleus 2, *VN* vegetative nucleus of the pollen tube. Bar = 10 μm

images, indicating the presence of the degenerated synergid contents after the entry of a pollen tube (Fig. 1c, f).

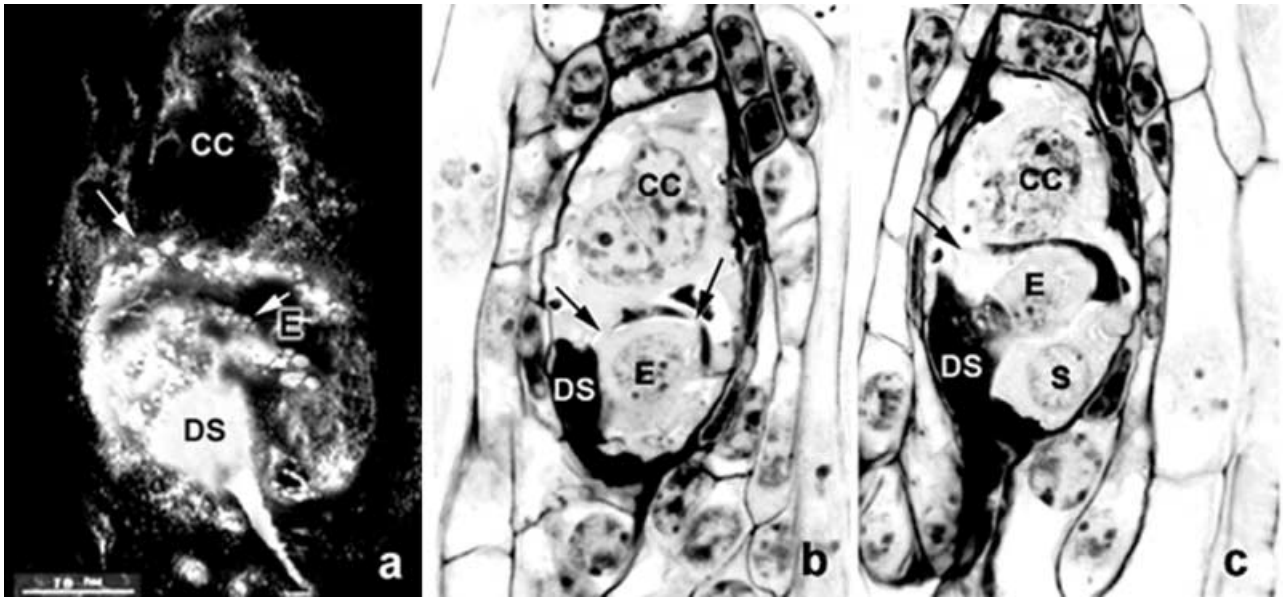
Actin staining shows a strong fluorescence signal within the degenerating synergid and in the space between cells (Fig. 2a). The flow of the synergid contents is interrupted at points where there are areas of ''tight'' association between the egg cell and the central cell (Fig. 1h; Fig. 2b, c). It is important to note that the flow of the synergid contents begins prior to sperm movement (Fig. 1d–f). The green fluorescence signal fades with the completion of the fertilization process.

A large amount of actin material is present in the degenerated synergid after pollen tube entry (Fig. 2a). At this time, the bright fluorescence of actin material prevents a clear visualization of the sperms. The staining intensity soon fades due to a reduction of labeled actin within the degenerated synergid, disclosing the structural features of the sperms as well as the egg cell and the central cell (Fig. 3a). The sperms are pear-shaped with pointed tails. The actin filaments are prominent within the sperms. The nuclei of the sperm cells also assume a similar shape (Fig. 3b).

Gamete fusion

At the time of gamete fusion, the egg cell takes on a characteristic shape. A small extension is present on the side near the sperm (Fig. 3c, d). Such an extension creates a depression to receive the sperm. As the sperm enters the egg cell cytoplasm, initially its ''tail'' is still visible within the cytoplasm of the egg cell (Fig. 3e, f), indicating that the cytoplasmic mass of the sperm has also entered the cytoplasm. At this time, the nucleus of the egg cell elongates (Fig. 4a) and actin is extremely

Fig. 2a–c The distribution of cytoplasmic content from the degenerated synergid of Nun orchid. **a** Bright actin coronas (*arrows*) appear after the penetration of the pollen tube into the degenerated synergid. The lower corona band is directed towards the egg cell and the upper band towards the central cell. This micrograph was taken using a whole-mounted isolated embryo sac. **b, c,** Adjacent longitudinal plastic sections of an embryo sac showing the contents of the degenerated synergid. The flow of the synergid content is not even over the entire surface of the egg cell and the central cell because the content is interrupted by ''tight spots'' (*arrows*) between the cells. *CC* central cell, *DS* degenerated synergid, *E* egg cell, *S* persistent synergid. Bar = 10 μm



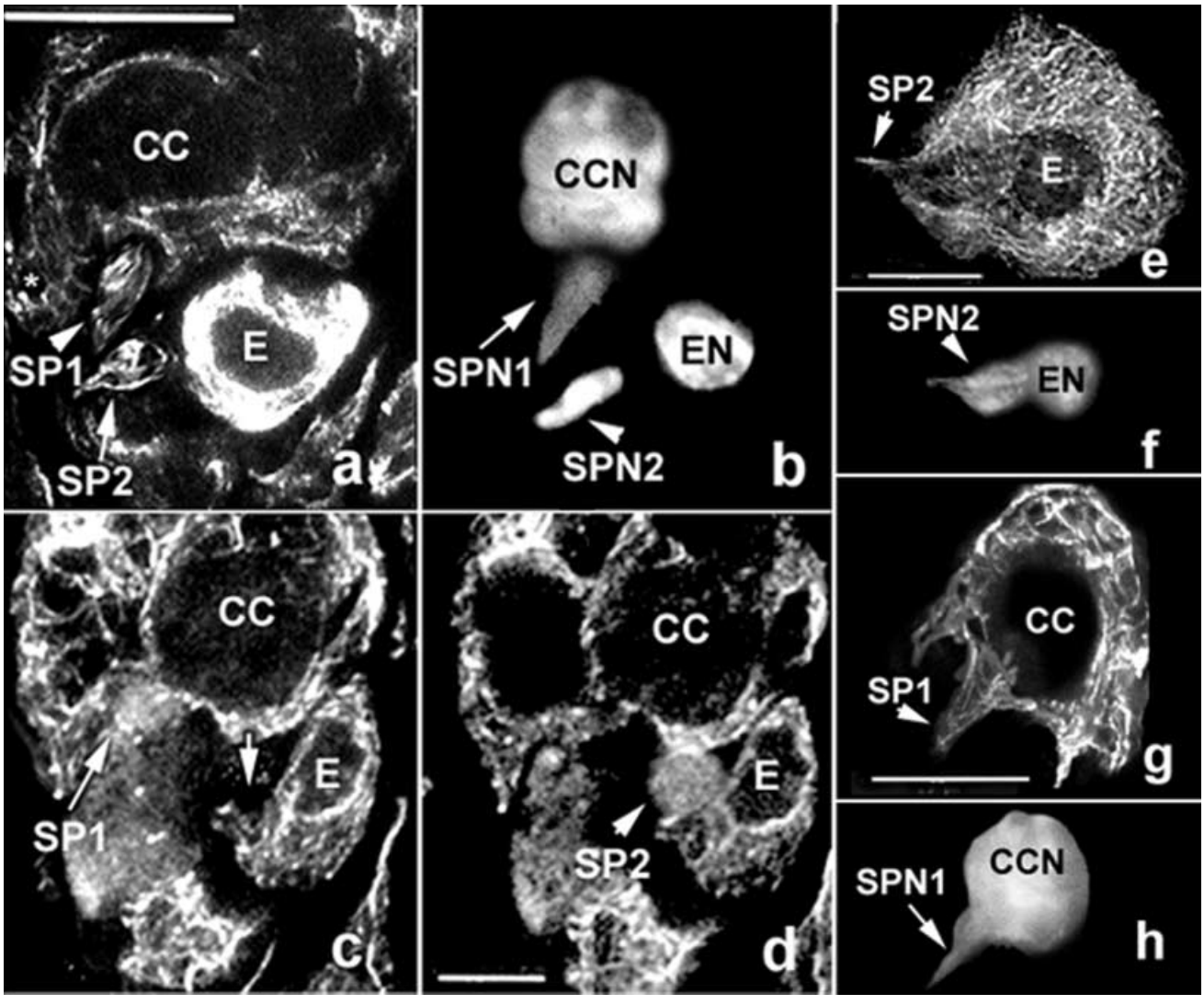


Fig. 3a–h The actin cytoskeleton of Nun orchid within the cells of the embryo sac during fertilization. **a** The actin filaments mark the boundaries of various cells. The sperm cells are pear-shaped with a pointed tail. One sperm is moving towards the central cell. Near the point of entry, the corner of the central cell extends towards the sperm, and actin filaments (*) are abundant near the site of sperm entry. The other sperm moves towards the egg cell. The egg cell has a high concentration of actin and fluoresces strongly. **b** Corresponding nuclei of the cells in **a**. **c, d** Two different optical sections of the same embryo sac. The location where sperm cell 2 binds with the egg cell has a characteristic extension (*arrow*). Sperm cell 1 is located near the central cell. **e** During embryo sac isolation, individual cells occasionally separate from one another. Sperm cell 2 has penetrated into the egg cell and its tail is still clearly visible within the egg cell cytoplasm. **f** Corresponding DAPI image of the nucleus of sperm cell 2 and the egg nucleus. **g** Actin filaments in the central cell are less abundant than in the egg cell. However, the corners of the central cell are clearly marked by actin filaments. The tail of sperm cell 1 is still clearly visible as it enters the central cell. **h** Corresponding position of the nuclei in the cell shown in **g** *CC* Central cell, *CCN* central cell nucleus, *E* egg cell, *EN* egg cell nucleus, *SP1* sperm cell 1, *SPN1* sperm cell 1 nucleus, *SP2* sperm cell 2, *SPN2* sperm cell 2 nucleus. Bars = 25 μm (**a, b, e–h**), 10 μm (**c, d**)

abundant, surrounding both nuclei, as indicated by the bright fluorescence (Fig. 4b, c). However, this actin appears more granular in appearance. Once the nuclei unite, the zygote nucleus returns to a spherical shape and a filamentous form of actin reappears (data not shown).

For the formation of the endosperm mother cell, the sperm targets the corner of the central cell near the tip of the degenerated synergid (Fig. 3a, c; Fig. 4b, c). In the vicinity of where the sperm enters, an extension of the central cell forms and appears to ''engulf'' the sperm (Fig. 3a). Actin filaments are abundant within the cell extension (Fig. 3a, c). The nucleus of the central cell is located near the site of sperm entry. As the sperm enters the central cell, similar to the egg cell, a distinct sperm tail is present in the central cell cytoplasm (Fig. 3g, h). Prior to fusion of the nuclei, the central cell nucleus becomes greatly elongated and fluoresces brightly (Fig. 4a). After fusion of the nuclei, the endosperm nucleus returns to a spherical shape. As in many other orchids, the endosperm degenerates soon after.

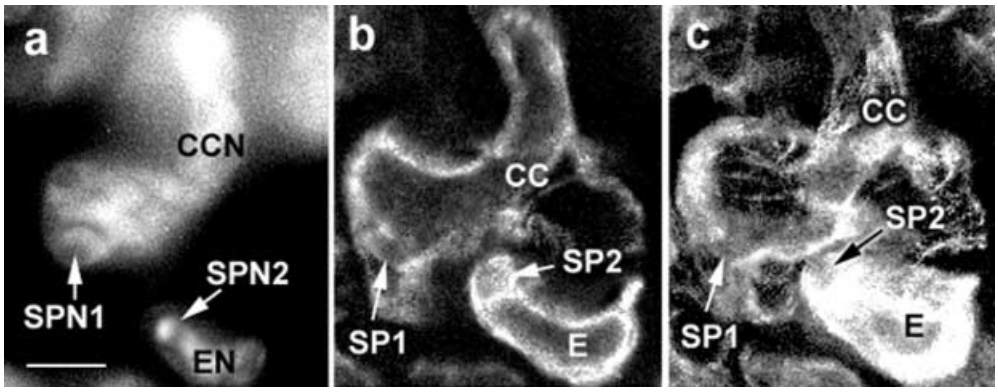


Fig. 4a–c Nuclear morphology and actin distribution after sperm entry in Nun orchid. **a** The nuclei of both the egg cell and the central cell become elongated at the time of gamete fusion. Both sperm nuclei are found within the cytoplasm of their respective partners. **b, c** Optical sections of the same embryo sac showing the abundance of actin filaments within the cytoplasm of the cells, especially around the nuclei. *CC* Central cell, *CCN* central cell nucleus, *E* egg cell, *EN* egg nucleus, *SP1* sperm cell 1, *SPN1* sperm cell 1 nucleus, *SP2* sperm cell 2, *SPN2* sperm cell 2 nucleus. Bar = 10 μm

Discussion

This study demonstrates the advantages of using orchid ovules as an experimental tool to study fertilization in plants. In orchids, a successful pollination event triggers ovule development (Yeung and Law 1997). Numerous pollen tubes grow towards the developing ovules and fertilization can only take place after the ovules have matured. In the Nun orchid, ovule development requires approximately 38 days after pollination. This long duration allows us to pinpoint the timing of pollen tube entry into the mature ovules. With the numerous fertilized ovules produced within a single ovary and long duration of the fertilization process, we were able to capture events related to sperm movement and fusion with their respective target cells.

It is well known that during the course of fertilization, one of the synergids begins to degenerate in preparation for pollen tube entry (see Russell 1992). The degeneration of a synergid generates new physical and chemical environments that enable the sperms to move and target their respective mates. These naturally transient steps are prolonged in orchids, permitting a better understanding of changes within the degenerated synergid.

It is generally known that the "cytoplasmic mass" of the degenerating synergid is unique, as it contains a variety of organelles, and it has a high actin content (Russell 1992; Huang and Russell 1994). In the Nun orchid, a large amount of actin is present, as judged by the bright fluorescence after rhodamine-phalloidin staining. Furthermore, a bright green fluorescence of the synergid is also observed after DAPI staining of the isolated embryo sac. Although the exact nature of this fluorescence is not known, the DAPI stain must interact with components of the degenerated synergid to produce this. Fortuitously, the presence of this fluorescence enables us to study the inter-relationship between the flow of the synergid content and the movements of the sperm nuclei at the same time. The simultaneous viewing of the green fluorescence and various nuclei within the embryo sac indicate that the synergid content begins to expand prior to sperm separation and movement. Two main channels are formed, one towards the direction of the egg cell and the other towards the central cell. Judging from the light-microscopic observations and actin staining, these are the actin coronas as reported in other species. The formation of actin coronas is one of the key features observed at the time of fertilization and appears to be a universal phenomenon (see Russell 1996). In tobacco, two actin coronas appear at the interface of the synergid, egg cell, and central cell (Huang and Russell 1994). In maize, the formation of actin coronas occurs prior to fertilization and could be detected prior to pollen tube entry (Huang and Sheridan 1998). The formation of actin coronas at the time of fertilization leads to the suggestion that they play a role in sperm movement. The actin coronas could serve as a guide or provide a pathway for the proper targeting of sperms to their respective partners (Huang and Sheridan 1998; Huang et al. 1999). In the Nun orchid, actin coronas form after pollen tube entry but prior to sperm separation and movement. The expansion of the synergid content may generate forces that aid in sperm separation and movement. From the fluorescence pattern of isolated embryo sacs and plastic sections, we noted that the flow of the synergid content is interrupted at points where there are areas of "tight" association between the egg cell and the central cell. These areas may represent the symplastic communication channels between the central cell and the egg apparatus cells, as described in the embryo sac of *Torenia fournieri* (Han et al. 2000). The presence of "loose and tight junctions" between cells may have created pre-determined channels that allow for the proper channeling and targeting of the sperms to their respective sites of entry.

Little information is available concerning the actin cytoskeleton in plant sperm cells. Direct experimental evidence indicating the presence of actin in sperm cells

is lacking. As a result, Palevitz and Liu (1992) conclude that F-actin is absent from the generative cells and sperm cells in plants. In *Epidendrum scutella*, intranuclear microfilaments are present in the sperm cells within the pollen tube, suggesting the presence of actin within sperm cells (Cocucci 1988). This study clearly reveals the presence of actin within the sperms as well as actin within the egg cell and the central cell. The sperms are pear-shaped with pointed tails and actin filaments oriented along the long axes of the cells. Although microtubules have not been labeled in this study, it is clear from other reports that they are also present in the sperms (Southworth and Cresti 1998). Thus, it is likely that with the presence of both cytoskeletal elements in the sperm cell (Zhang and Russell 1999), sperm movement within the degenerating synergid could be in part a self-regulated process. The material within the degenerating synergid could serve as a special milieu interacting with the sperms and resulting in the proper targeting of sperms to their respective sites (Russell 1997). It is important to note that changes in actin organization occur during the course of the fertilization event in *Torenia fournieri* (Fu et al. 2000) and a reduction in actin fluorescence also occurs in the Nun orchid. These observations indicate changes in the composition of the ''cytoplasmic'' mass of the degenerated synergid during the course of fertilization events. Thus, further chemical characterization of the synergid content and immunochemical localization of motor proteins such as myosin may provide further insights into sperm movement within the degenerated synergid.

The presence of a projection from the egg cell and the extension from the corner of the central cell at the time of sperm entry suggest active interactions between male and female gametes that lead to cell fusion. During the course of this study, we noted that the points of sperm entry into their respective target cells are very consistent between preparations. That is, for the central cell, the point of entry is at the projection near the end of the degenerating synergid content. For the egg cell it is at the corner where the cells meet. This consistency suggests that positionally pre-determined regions are present on the surface of the egg cell and the central cell, and these regions are responsible for ''receiving'' the sperm cells. Recent studies using in vitro methods (Kranz 2001; Sun et al. 2001) to study plant fertilization should further our understanding of the mechanism of gamete fusion in flowering plants.

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