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Actin coronas in normal and *indeterminate gametophyte1* embryo sacs of maize

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Abstract The actin cytoskeletal organization and nuclear behavior of normal and *indeterminate gametophyte1* (*ig1*) embryo sacs of maize were examined during fertilization. After pollination, during degeneration of one of the synergids and before arrival of the pollen tube, the cytoskeletal elements undergo dramatic changes including formation of the actin coronas at the chalazal end of the degenerating synergid and at the interface between the egg cell and central cell. The actin coronas are present only for a limited period of time and their presence is coordinated with pollen tube arrival and fusion of the gametes; they disappear before the zygote divides. This allows us to estimate the frequency of fertilized ovules along the ear. Up to 88% of the ovules on an ear contain actin coronas in the embryo sacs when observed 16–19 h after pollination, indicating the high frequency of fertilizing kernels along the ear at this stage. In the *ig* embryo sacs, two or more degenerated synergids containing actin coronas at their chalazal ends receive multiple pollen tubes for gametic fusion and can consequently give rise to twin or polyembryos. These findings with the monocot maize are consistent with previous reports on the dicots *Plumbago* and *Nicotiana*, suggesting that the formation of actin coronas in the embryo sac during fertilization is a universal phenomenon in angiosperms and is part of a mechanism of interaction between gametic signaling and actin cytoskeleton behavior which appears to precisely position and facilitate the access of male gametes to the egg cell and central cell for fusion.

Key words Fertilization · Maize · Actin · *ig* mutant

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Introduction

In flowering plants, sexual reproduction involves a complex sequence of events leading to formation of the zygote and endosperm in a process known as double fertilization (for reviews see Jensen 1974, 1998; Russell 1992). The pollen tube deposits two sperm cells into the embryo sac and these subsequently fuse with the egg and the central cell. The pollen tube, apparently guided by the physical and chemotropic geometry of the ovule, penetrates the embryo sac. The exact site of pollen tube lysis and where the two male gametes are released also appear highly regulated. It is presumed that the two female targets, the egg cell and the central cell, have a relatively narrow receptive period in vivo following pollination and that release of the non-motile male gametes must be precisely positioned. In most species, the prominent features in this process include degeneration of one of the synergids, its subsequent penetration by the pollen tube, discharge of the pollen tube cytoplasm into the synergid, release of the male gametes, their migration to their fusion sites, and sperm fusion with the female targets (Russell 1992; Dumas and Mogensen 1993).

The role of the cytoskeletal components in the cellular control of double fertilization merits detailed attention (Russell 1993). There is considerable information regarding the pollen tube and its contents indicating that cytoskeletal elements are involved in pollen tube elongation, organelle transport, and the descent of the generative and sperm cells (Palevitz and Tiezzi 1992; Pierson and Cresti 1992). Interestingly, the male gametes and their progenitor generative cells appear to lack actin (Palevitz and Liu 1992; Palevitz and Tiezzi 1992). The movement of male germ cells probably occurs as a consequence of intimate interactions between the actin cytoskeleton of the pollen tube and myosin-like proteins on the surface of the male gamete lineage (Heslop-Harrison and Heslop-Harrison 1989a; b; Tang et al. 1989). The presence of kinesin (Cai et al. 1990) and dynein heavy chains (Moscatelli et al. 1995) suggests that other locomotory proteins could also have a role in

pollen tube function. Current views hold that actin provides a dynamic scaffold for associated locomotor proteins.

During fertilization, both synergids are initially intact before pollen tube arrival (Huang and Russell 1992). Breakdown of the plasma membrane and vacuole of the receptive synergid occurs at pollen tube arrival, when the high concentration of calcium present in the synergid vacuole (Chaubal and Reger 1990, 1992a, b) is released as membrane-associated calcium (Huang and Russell 1992). Microtubules and actin in the synergid are apparently depolymerized during synergid degeneration and do not appear to impede pollen tube arrival and discharge. At this stage, two actin coronas occur at the interface between synergid, egg and central cell in *Nicotiana tabacum* (Huang and Russell 1994) and an actin corona is present at the interface between the egg and central cell in synergidless *Plumbago zeylanica* (Huang et al. 1993). The coronas appear to trace the pathways taken by the sperm cells during their passage to the egg cell and the central cell.

The presence of the actin coronas in the embryo sacs during the fertilization process in *Nicotiana* and *Plumbago* raises the following questions concerning the role of the cytoskeleton during fertilization. (1) How is the degeneration of the receptive synergid controlled and what is its role in acceptance of the male gametes? (2) How do the male gametes move within the synergid and migrate to their female target cells? (3) How does the cytoskeleton coordinate this migratory process? (4) Is the presence of actin coronas a universal phenomenon in embryo sacs during fertilization of angiosperms?

In this study we used fluorescent light microscopy to examine the fertilization events in wild-type and *indeterminate gametophyte* (*ig*) mutant embryo sacs in maize to address some of the aformentioned questions. We observed the presence of similar actin coronas during fertilization in wild-type and *ig* mutant embryo sacs. These findings, along with our earlier studies with *Plumbago* and *Nicotiana*, strongly suggest that the actin cytoskeleton within the embryo sac is strategically repositioned to play an important role in the dynamic process of pollen tube arrival, pollen tube discharge, gametic fusion, and nuclear fusion. Formation of the actin corona in the embryo sacs of both dicots examined previously, as well as the occurrence of this process in the monocot maize, suggest that it may be a ubiquitous and necessary step to secure gametic fusion in angiosperms.

Materials and methods

Plant materials

Seeds of Inbred line A619 and F1 of A158/M14 (*ig/ig*) were grown in the greenhouse under 16 h of light. Pollination was carried out according to the procedure described previously (Neuffer 1994). The ears were collected at different times after pollination.

Chemical fixation and plastic thick sectioning

Ovaries were dissected and fixed in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 5 min under vacuum and further fixed in glutaraldehyde for 4 h at room temperature and 1% osmium tetroxide at 4°C overnight. Tissue was embedded in Spurr's resin and then sectioned at 4-µm thickness. The sections were stained with 1% toluidine blue O. Observation of sections was conducted using a Laborlux S (Leitz, Germany) equipped for differential interference contrast (DIC).

Double labelling of actin and DNA in unfixed and fixed embryo sacs

Ovules were dissected and placed in PEMG buffer (50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA and 4% glycerol at pH 6.8) containing 2% DMSO. The materials were incubated in enzyme solution consisting of 1% cellulysin, 1% pectinase, and 0.3% pectolyase for 30 min. Embryo sacs were isolated as previously described (Huang and Sheridan 1994) and stained with 0.66 µM rhodamine-phalloidin and 0.5% Hoechst 33258 for 1 h. For the fixed material, ovules were fixed in a mixture of 4% paraformaldehyde, 2% DMSO in PEMG buffer for 3 h prior to isolation of embryo sacs. Materials were digested by the enzyme solution and stained with rhodamine-phalloidin and Hoechst 33258 as described above. Light microscopy was conducted using a Leitz Laborlux S microscope equipped for epifluorescence microscopy.

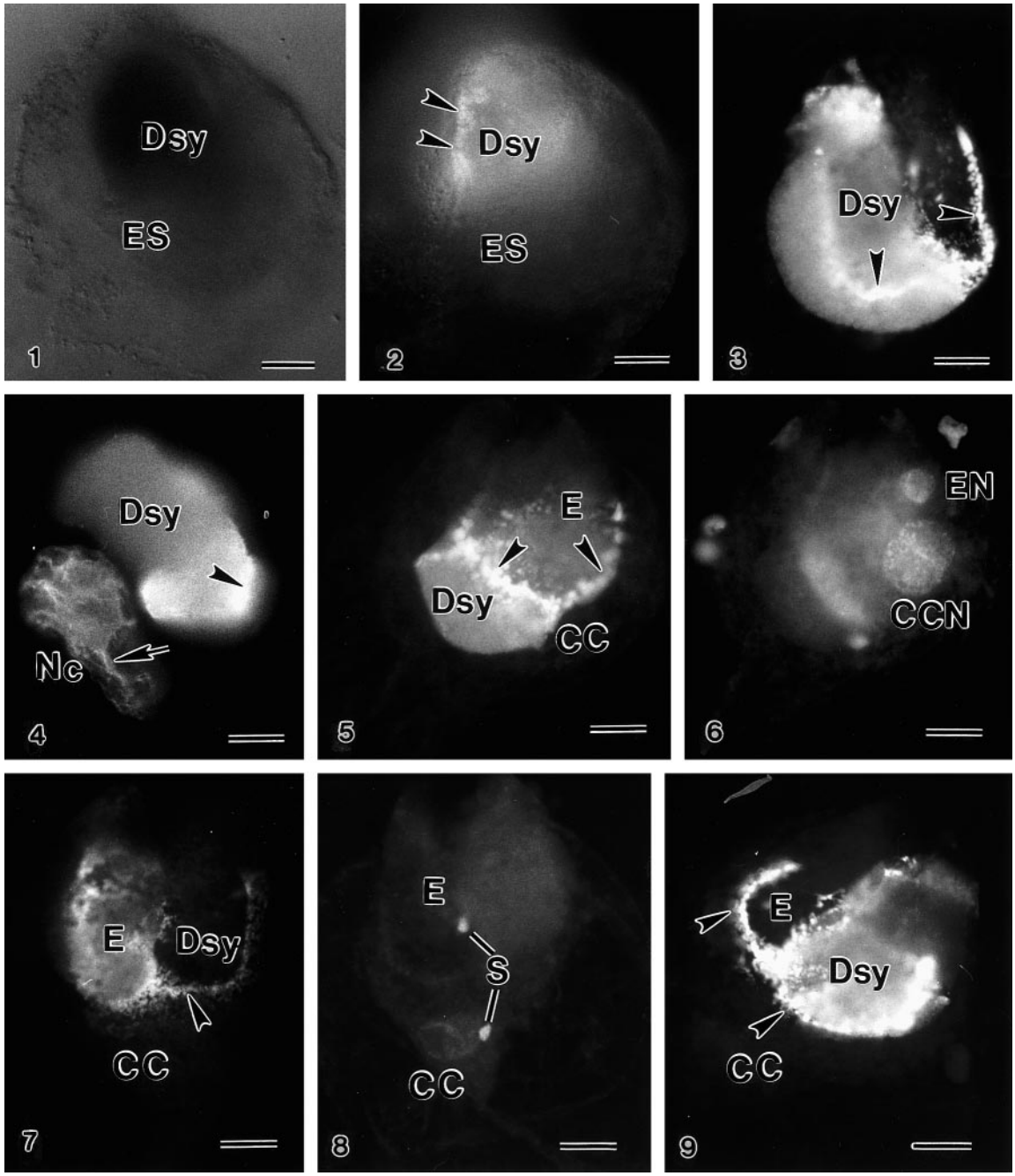
Results

The organization of actin and nuclear DNA during fertilization in normal embryo sac

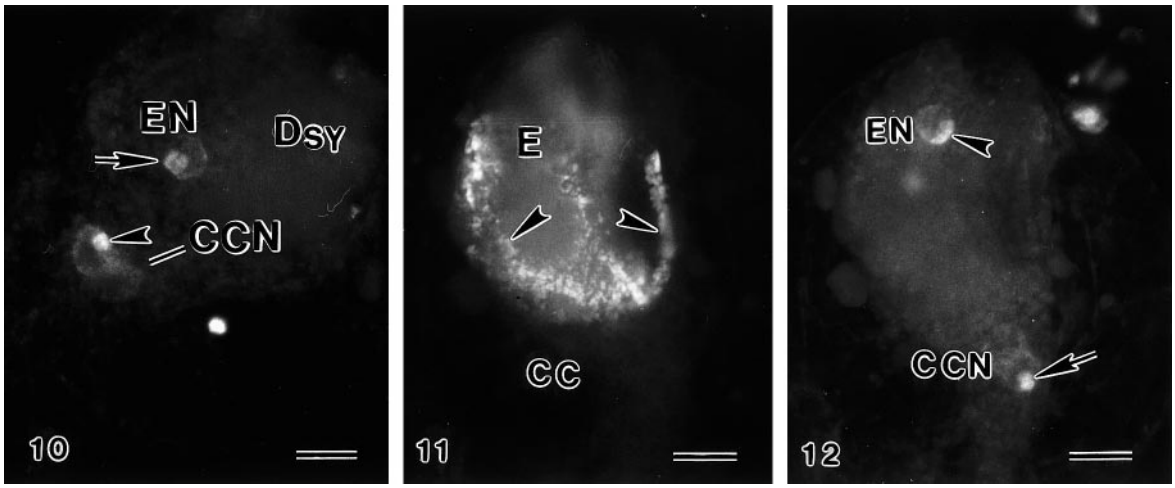
The time course of double fertilization in maize varies with growth conditions, particularly environmental temperature (Mól et al. 1994). It is important to precisely determine a time course for fertilization events in order to perform in vitro cytological and molecular studies. We determined the time course of fertilization in maize using the embryo sac isolation technique and double staining of actin and DNA. Our results are consistent with previous observations of fertilization events in *Plumbago* and *Nicotiana* which demonstrated that actin coronas are present only immediately prior to or during pollen tube penetration of the embryo sac and fertilization, but disappear before division of the zygote (Huang et al. 1993; Huang and Russell 1994). Since the occurrence of an actin corona is a unique phenomenon in the process of fertilization, it allows us to use this as a marker to estimate the frequency of fertilized ovules along the ear at different times after pollination (Table 1).

Table 1 Frequency of embryo sacs (ES) with actin coronas (AC) at different times after pollination. *HAP* Hours after pollination

HAP (h)	Number of ES (%) having AC	Number of ES (%) without AC	Total
0	0	45 (100%)	45
12	16 (28.6%)	40 (71.4%)	56
16	36 (81.8%)	8 (18.2%)	44
19	56 (87.5%)	8 (12.5%)	64
32	0	15 (100%)	15



Figs. 1–9 Localization of actin and nuclear DNA during fertilization in maize. *Bars*=2 µm. **Fig. 1** An isolated unfixed embryo sac (*ES*) 16 h after pollination. Note the *dark areas* in the micropylar end of the embryo sac identifying a degenerated synergid (*Dsy*). **Fig. 2** Rhodamine-phalloidin staining of actin in the same embryo sac (*ES*) shown in **Fig. 1** reveals dense labeling (*arrowheads*) at the chalazal periphery of the degenerated synergid (*Dsy*). **Fig. 3** Fixed degenerated synergid (*Dsy*) showing chalazal actin aggregates (*arrowheads*). The synergid was isolated 16 h after pollination. **Fig. 4** Actin labeling in the non-fixed degenerated synergid (*Dsy*) and a nucellus cell (*Nc*). Note that actin coronas (*arrow and arrowhead*) at the chalazal region of the synergid and actin bundles of the nucellar cells extend from the perinuclear region to the cell cortex. The synergid and nucellus cell were isolated at 16 h after pollination. **Fig. 5** Brightly-labeled actin aggregates (*arrowheads*) form actin coronas in the chalazal region of the degenerated synergid (*Dsy*) at its border with the egg cell, and between the egg cell (*E*) and the central cell (*CC*). The embryo sac was isolated 16 h after pollination. **Fig. 6** Localization of egg nucleus (*EN*) and central cell secondary nucleus (*CCN*) in the same embryo sac shown in **Fig. 5** by staining with Hoescht 33258. **Fig. 7** Actin aggregates of coronas (*arrowhead*) at the interface of the egg cell (*E*) and the central cell (*CC*) and at the chalazal region of the degenerated synergid (*Dsy*). The embryo sac was isolated 18 h after pollination. **Fig. 8** Localization of the two sperm nuclei (*S*) during their approach to fuse with the egg cell (*E*) and the central cell (*CC*) in the same embryo sac shown in **Fig. 7**; staining with Hoescht 33258. **Fig. 9** Actin aggregates of coronas (*arrowheads*) at the interface of the egg cell (*E*) and the central cell (*CC*) and at the chalazal-most region of the degenerated synergid (*Dsy*). The embryo sac was isolated 18 h after pollination



Figs. 10–12 Localization of actin and nuclear DNA during karyogamy. *Bar*=2 µm. **Fig. 10** One sperm nucleus (*arrow*) fusing with egg nucleus (*EN*) and second sperm nucleus (*arrowhead*) fusing with central cell secondary nucleus (*CCN*) in the same embryo sac shown in **Fig. 9**; staining with Hoescht 33258. **Fig. 11** Two actin coronas (*arrowheads*) are still present during the course of karyogamy. The embryo sac was isolated 18 h after pollination. *E*, Egg; *CC*, central cell. **Fig. 12** In the same embryo sac shown in **Fig. 11**, one sperm nucleus is fusing with the egg nucleus (*EN*) (*arrowhead*) and another sperm nucleus is fusing with the central cell secondary nucleus (*CCN*) (*arrow*). Note that the secondary nucleus partially retains the profiles of the two polar nuclei that are fusing to form it. The egg nucleus and central cell secondary nucleus appear to be migrating in opposite directions during their fusion with the sperm nuclei; staining with Hoescht 33258

Actin coronas and synergid degeneration

About 28% of the embryo sacs contained actin coronas when examined 12 h after fertilization. The frequency of embryo sacs containing an actin corona and male gametes rapidly increases 16 h after pollination, reaching about 87.5% by 19 h after pollination (Table 1). The isolated viable embryo sac manifests a dramatic change 16 h after pollination, as characterized by the darkened appearance of one of the two synergids (Fig. 1). Actin aggregates accumulate at the chalazal periphery of the degenerated synergid (Fig. 2), and some of the actin aggregates infiltrate into the intercellular space between the egg cell and central cell (Fig. 3). The isolated degenerated synergid exhibits a different actin pattern from that of nucellus cells. Dense-staining actin is distributed mainly at the chalazal end of the degenerated synergid while radiate actin bundles extend from the nucleus to the cortex of the nucellus cell (Fig. 4).

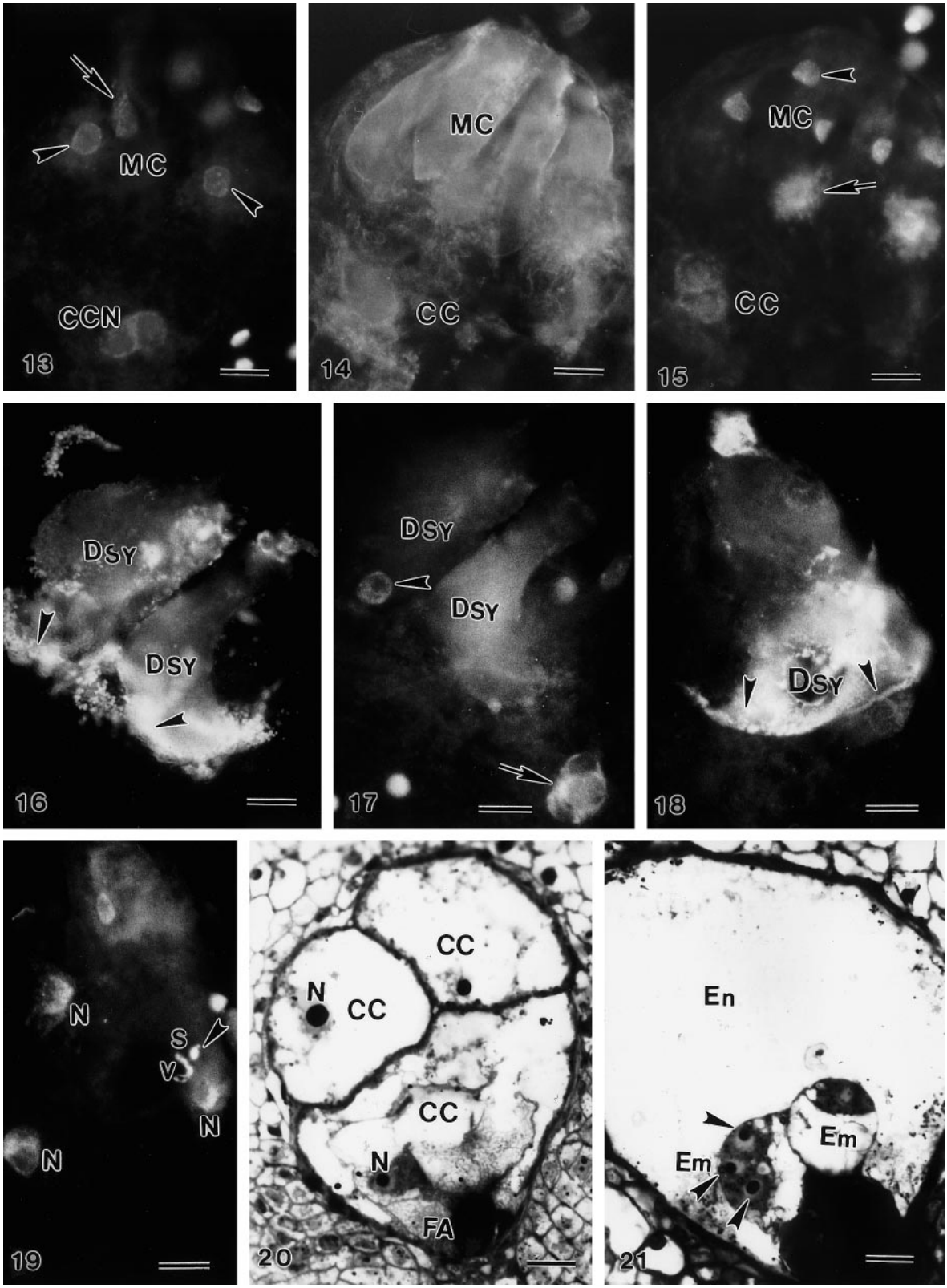
Actin coronas and gametic fusion

The brightly-labeled actin aggregates also form an actin corona at the interface between the egg cell and central cell (Figs. 5, 6). When the pollen tube arrives at the receptive synergid, it discharges two sperm cells. The sperm cells appear to approach the egg and central cells along the actin corona (Figs. 7, 8). Karyogamy was observed to occur 16–19 h after pollination (Figs. 9, 10). Frequently, the two sperm cells fuse synchronously with the egg cell nucleus and central cell secondary nucleus (Figs. 10, 12). The two actin coronas are still visible while the egg nucleus and the secondary nucleus of the central cell migrate in opposite directions during gametic fusion (Figs. 11, 12). The actin coronas remain in the embryo sac for several hours after gametic fusion but they disappear by 32 h after pollination and before the zygote divides (Table 1).

The organization of actin and nuclear DNA in the *ig* embryo sac during fertilization

The mature *ig* embryo sac contains variable numbers of micropylar nuclei in varying positions. The nuclei at the micropylar-most region become elongated and are presumably synergid nuclei. More than one putative egg nucleus is frequently observed and regularly two or more polar nuclei have fused to form the central cell secondary nucleus (Fig. 13). Actin appears to be present in the micropylar cells (Fig. 14). Seven nuclei are frequently seen in the micropylar end of the embryo sac, five of which

Figs. 13–19 Organization of actin and DNA in the *ig* embryo sac during fertilization. *Bars*=2 µm. **Fig. 13** A mature *ig* embryo sac containing five micropylar nuclei (*MC*) (two are slightly out of focus) with the two polar nuclei fusing together to form the central cell secondary nucleus (*CCN*). Note that one elongated nucleus at the micropylar-most end (*arrow*) is presumably a synergid nucleus and the other two nuclei (*arrowheads*) appear to be egg cell nuclei; staining with Hoescht 33258. **Fig. 14** Rhodamine-phalloidin staining of the micropylar cells (*MC*) in a mature *ig* embryo sac showing the distribution of actin. *CC*, Central cell. **Fig. 15** Seven micropylar nuclei in the same embryo sac shown in **Fig. 14**. Five small nuclei (one is labeled with an *arrowhead*) are presumably synergid nuclei and the other two are presumed egg nuclei (one is labeled with an *arrow*). Note the larger size of the presumed egg cell nuclei and the abundance of organellar DNA surrounding each of them. *CC*, Central cell. **Fig. 16** Numerous actin aggregates accumulate at the chalazal region of two degenerated synergids (*Dsy*) (*arrowheads*) in an *ig* embryo sac 19 h after pollination. **Fig. 17** Hoechst staining of the nucleus of one of the degenerated synergids (*Dsy*) (*arrowhead*) and the central nucleus (*arrow*) in the same embryo sac shown in **Fig. 16**. **Fig. 18** Dense staining of



actin at the chalazal region of a degenerated synergid during fertilization in an *ig* embryo sac (*arrowheads*) 19 h after pollination. **Fig. 19** In the same embryo sac shown in **Fig. 18** one of the sperm cells (*S*) (*arrowhead*) approaches to fuse with one of the micropylar nuclei. Note the elongated vegetative nucleus (*V*) close to the sperm nucleus (*S*). *N*, Nuclei. **Figs. 20–21** Longitudinal sections of the *ig* embryo sacs 51 h after pollination. *Bars*=2 µm. **Fig. 20** Longitudinal plastic section of a mature *ig* embryo sac showing three central cells (*CC*). *N*, Nuclei; *FA*, filiform apparatus. **Fig. 21** Longitudinal plastic section of an *ig* embryo sac fixed 51 h after pollination showing twin embryos (*Em*). Note one embryo contains three nuclei (*arrowheads*). *En*, Endosperm

are at the micropylar-most region and are presumably synergid nuclei while the other two are putative egg cell nuclei (Fig. 15).

Presence of multiple actin coronas and multiple degenerated synergids in *ig* embryo sacs

Similar to normal embryo sacs, one or more synergids degenerate before pollen tubes penetrate and actin coronas are also present at the chalazal end of the degenerated synergids in *ig* embryo sacs (Figs. 16, 17). Some *ig* embryo sacs contain two or more degenerated synergids and have attracted two or more pollen tubes. The actin aggregates accumulate at the chalazal end of the degenerated synergid (Fig. 18) while the sperm cells approach the female target cells for fusion (Fig. 19). Polyembryony is frequently seen after fertilization. Interestingly, some mature *ig* embryo sacs contain three central cells (Fig. 20) and some fertilized *ig* embryo sacs have two embryos (Fig. 21).

Discussion

Presence of actin coronas during fertilization may be ubiquitous for gametic fusion in angiosperms

Our results are in agreement with the previous observations of fertilization events in *Plumbago* and *Nicotiana* (Huang et al. 1993, Huang and Russell 1994) and demonstrate that actin coronas are present prior to pollen tube penetration of the maize embryo sac, but disappear before nuclear division of the zygote. This enables us to use actin coronas as markers to estimate the frequency of fertilized ovules along the ear very soon after pollination. The distribution of kernels along the maize ear that are receptive for pollination and fertilization is correlated with the maturity and functional capacity of their female gametophytes (Huang and Sheridan 1994). Ovules that contain actin coronas are found at a high frequency at the middle and bottom of the ear when pollinated at the time that silks emerge. This result is consistent with the previous report that a high yield of fertilized kernels was obtained when kernels removed from the middle and bottom of an ear were pollinated in vitro at the time that silks emerged from the ear (Dupuis and Dumas 1989, 1990). Ovules in the upper region of an ear are less developed and are fertilized later than those located below (Huang and Sheridan 1994).

During degeneration of the receptive synergid and before arrival of the pollen tube, cytoskeletal elements undergo dramatic changes that include depolymerization of microtubules and formation of two actin coronas at the chalazal end of the synergid and at the interface between the egg and central cell, respectively (Huang and Russell 1994). Interestingly, the embryo sac of *Plumbago*, a plant that lacks synergids, contains only a single actin corona at the interface between the egg and central cell (Huang et al. 1993). The localization of actin in both the fixed and non-fixed isolated embryo sacs and synergids of maize confirms that actin normally aggregates at the chalazal periphery of the degenerated synergid and is not a result of the fixation process. Formation of actin coronas prior to pollen tube penetration means that the coronas are not the products of the pollen tube cytoplasm.

The appearance of the corona as a band of tightly-packed but distinct granules of rhodamine-phalloidin stained aggregates was confirmed earlier using transmission electron microscopy. The corona bands are composed of patches of electron-dense material approximately 0.2–1.5 μm in size that are essentially homogeneous. Immunogold labeling indicated that the coronas are rich in actin. In tobacco, two coronas are evident (Huang and Russell 1994); this has been confirmed using electron microscopy. Here we report that these two coronas have also been found in maize using fluorescent light microscopy. The most micropylar of the two corona bands begins at the mid-lateral region of the degenerating synergid and extends to the chalazal end, whereas the other band forms along the side of the egg cell and extends along its chalazal boundary with the central cell.

Actin coronas in tobacco and maize appear to be positioned along the expected pathway that gametes would follow after being discharged from the pollen tube (Huang and Russell 1994; this report). Although the function of the actin coronas is still unresolved, the timing of their formation and their disposition at the edge of the egg cell and the degenerated synergid suggest that they are directly involved in transmission of the male gametes for gametic fusion as they exit the receptive synergid. In support of this is the observation that the synergidless embryo sac of *Plumbago zeylanica* has only one corona located at the interface of the egg cell and central cell. The sperm cells are deposited adjacent to the pollen tube tip and transported a short distance for gametic fusion (Russell 1983). The location of the actin coronas along the path of the gametes is consistent with the idea that the coronas play a role in sperm migration rather than being simply a degradation product of the synergid cytoplasm.

The presence of actin coronas in the maize *ig* embryo sac during fertilization provides additional evidence of involvement of actin during fertilization. Actin coronas were found mainly at the chalazal ends of degenerating synergids. Frequently, two or more degenerating synergids occur in the *ig* embryo sac and appear to attract two or more pollen tubes for gametic fusion during fertilization. Release of multiple pairs of male gametes and their fusion with multiple eggs results in the formation of multiple embryos. According to previous reports, the indeterminate number of micropylar and central nuclei in *ig* embryo sacs results in an indeterminate number of egg cells and central cell nuclei as well as the variable ploidy level of endosperm that is characteristic of this mutant (Kermicle 1971; Lin 1978; Huang and Sheridan 1996). Consequently, the *ig* mutation gives rise to polyembryony, heterofertilization and an elevated ploidy level of

the endosperm after fertilization (Kermicle 1971; Lin 1978). In *ig* embryo sacs, twin embryos were regularly found and twins frequently were non-concordant if the marker genes were introduced paternally. Those twins identical in paternal inheritance were proposed to originate from the fertilization of two genetically identical eggs by the sperms of one male gametophyte (Kermicle 1971). Our results support the previous genetic analysis of this mutant. The association of multiple actin coronas with multiple degenerated synergids and multiple embryos in *ig* embryo sacs supports the proposition that actin coronas are a ubiquitous phenomenon and may play an important role in sperm migration within the embryo sac during fertilization in angiosperms.

Transitory appearance and precise positioning of actin coronas in the embryo sac suggest an interaction between gametes and the cytoskeleton

The dynamic features of the actin corona inside the embryo sac suggest a complex relationship between gametic signaling and the behavior of the actin cytoskeleton during double fertilization. During fertilization, both synergids are initially intact before pollen tube arrival but one has already begun to degenerate (Huang and Russell 1992). The plasma membrane and vacuole break down upon the arrival of the pollen tube, at which time high concentrations of calcium present in the synergid vacuole (Chaubal and Reger 1990, 1992a, 1992b) are released as membrane-associated calcium (Huang and Russell 1992). Our earlier observations in *Nicotiana* revealed that microtubules and actin in the synergid are apparently depolymerized during synergid degeneration and do not appear to impede pollen tube arrival and discharge (Huang and Russell 1994). At this stage, two actin coronas occur at the interface between synergids, egg and central cell. One important finding in our previous research is that the actin corona is apparently identical to the electron-dense bodies observed in rapid-freeze, freeze-substituted material labeled using immunogold (Huang et al. 1993; Huang and Russell 1994). These electron-dense bodies are similar to those seen between the cells of the female germ unit in many species (Russell 1992), suggesting that the actin coronas are a general feature of the embryo sac in angiosperms, including *Plumbago*, *Nicotiana* and maize (Huang et al. 1993; Huang and Russell 1994; Huang and Sheridan 1996). The actin corona is present only immediately before and during gametic fusion and disappears soon after fertilization. The corona appears to trace the pathway taken by the sperm cells during their passage to the egg cell and central cell. The occurrence of a myosin coating on the surface of the sperm cells in pollen tubes (Heslop-Harrison and Heslop-Harrison 1989a, b; Tang et al. 1989) may allow their active conveyance on the actin corona of the embryo sac.

The release of calcium ions from reservoirs within the receptive synergid at the time of fertilization may alter the physiology of the gametes and the behavior of the cytoskeleton and cytoskeleton-associated proteins. In vitro fertilization has demonstrated that calcium is required for fertilization (Faure et al. 1994). Calcium is known to modulate the activity of actin-associated proteins like profilin (Valenta et al. 1993; Sohn et al. 1994) and gelsolin (Janmey 1994). The original view of profilin as a "sequestering protein" has been replaced by models in which profilin can act either to stimulate polymerization or cause destruction, depending on cellular conditions (Tilney et al. 1983; Staiger et al. 1994; Drøbak et al. 1994). An embryo sac profilin might promote formation of the actin coronas, and a pollen tube profilin could lead to their destruction. Major issues to be resolved include determining the actin dynamics in the coronas, localizing profilin and calcium during corona formation, and determining how the actin coronas may interact with the sperm cells in vivo.

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