

Recent Progress in Research of the Mechanism of Fertilization in Angiosperms

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ABSTRACT

Fertilization in angiosperms is a complex process. When the pollen tube enters the degenerated synergid of an embryo sac, two sperm cells are released inside. The two sperm cells are connected in the pollen tube but after their release in the degenerated synergid, they must separate. One of the two sperm cells moves to the egg cell and fuses with it to form a zygote, the other one moves to the central cell to form the endosperm. The process of male and female gamete recognition is a critical interaction but remains poorly understood. In this review, we discuss recent progress in the study of the cell cycle of male and female gametes before fertilization, and the question of synergid degeneration. Herein we analyze the status of research on the movement of the two sperm cells in the degenerated synergid, and the phenomenon of signal reactions between male and female gametes. We also evaluate our progress in understanding the preferential fertilization of sperm cells and egg cell activation.

Keywords: angiosperms, egg cell, fertilization mechanism, sperm cell, zygote

CONTENTS

INTRODUCTION.....	35
THE CELL CYCLE OF GAMETES AND FERTILIZATION	35
THE FUNCTION OF DEGENERATED SYNERGIDS	37
SIGNALS BETWEEN MALE AND FEMALE GAMETOPHYTES	37
THE SPERM CELL TRANSPORT WITHIN THE EMBRYO SAC	38
PREFERENTIAL FERTILIZATION	38
EGG CELL ACTIVATION	39
CELLULAR DETERMINATION OF ZYGOTE.....	40
CONCLUSIONS AND PROSPECTS.....	40
ACKNOWLEDGEMENTS	40
REFERENCES.....	40

INTRODUCTION

Double fertilization in angiosperms is a key process of higher plant development and a theme of interest to many scientists, and has thus been the topic of many reviews (Antonie *et al.* 2001b; Faure and Dumas 2001; Lord and Russell 2002; Raghavan 2003; Weterings and Russell 2004). In angiosperms the embryo sac is embedded deep in the ovary, where the process of double fertilization occurs. The hidden location of this process makes research into the mechanism of fertilization difficult. As a result, many questions about the process remain to be answered. The results of studies on the mechanism of this process in lower plants and animals have led to some new techniques and concepts that can be utilized in the study of higher plants. Using isolated sperm and egg and referring to the studies of animals, we have an opportunity to solve age-old questions about angiosperm fertilization. In the current review, we discuss and analyze the progress of studies into the fertilization mechanism of angiosperms, the cell cycle of male and female gametes, the function of degenerated synergids, signaling between male and female gametes, sperm movement in degenerated synergids, and preferential fertilization and cel-

lular determination of zygotes. Each of these topics is covered briefly compared with previous reviews, but we have used the discussion to introduce our unique points of view.

THE CELL CYCLE OF GAMETES AND FERTILIZATION

Cell cycle regulation is important to the growth and development of plants. In the 1950's, the first examinations of plant gamete nuclei DNA contents were performed, using the Feulgen reaction. Preliminary results lead to the rule of DNA content of male and female gametes and zygotes, and indicated that most gametes of higher plants fuse at 1 C of relative content. The Feulgen reaction of DNA, however, is restricted by the state of nuclei and experimental conditions. In many plants, the egg cell at anthesis cannot be stained. In other reports, for example in barley (*Hordeum vulgare*), data indicating DNA contents of sperm cells, reported by different authors, were contradictory [1-2 C (Hesemann 1973) and 2 C (D'Amato *et al.* 1965)]. In *Hordeum distichum*, sperm cells contained 1 C DNA (Mericle and Mericle 1970). These and other difficulties lead to a decline in research pertaining to the DNA content of gametes, and fewer

published reports.

It is possible to measure the DNA content of male and female gametes and zygotes by following the development of isolated sperm and egg cells from higher plants using new DNA fluorescence dyes. The study of DNA content in gametes and zygotes has entered a new phase of development (Sherwood 1995; Mogensen and Holm 1995; Mogensen *et al.* 1999; Pönya *et al.* 1999). These authors examined the DNA content of plant sperm and egg cells and zygotes, using fluorescence dyes 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI) and H33258. Friedman (1991) measured the DNA content of male gametes of *Ephedra trifurca* using DAPI dye and combined fertilization events with the concept of the cell cycle, introducing the relationship between sperm development and the cell cycle, and proposing a model of the cell cycle during gametic fusion. They studied DNA synthesis in developing sperm cells of *Gnetum gnemon* and the relationship between the cell cycle and sexual reproduction in this gymnosperm (Carmichael and Friedman 1995). Friedman then examined the sperm DNA content of *Arabidopsis thaliana* and found that the two newly formed sperm cells enter the S phase of the cell cycle after mitosis of the generative cell, and the DNA content of both sperm cells then begins to increase. At the time of anthesis the DNA content of both sperm cells is 1.5 C. After pollination both sperm cells continue to synthesize DNA in the pollen tube and reach 1.75 C when they arrive at the ovary. Near the time of fertilization, the DNA content of the sperm cells can reach 1.98 C (Friedman 1999). This result indicates the sperm cells of *A. thaliana* begin to synthesize DNA in the pollen grain and both reach nearly 2 C prior to fusing with the egg cell and central cell. Both male and female gametes belong to the G₂ Type. However, Friedman could not measure egg cell DNA changes because the nuclei of mature egg cells have no fluorescence (Friedman, pers. comm.). These results lead us to question how the DNA content of eggs and central cells changes when sperm cells reach 2 C DNA before fusion with egg and central cells, and, especially, whether there are two polar nuclei. Although these results suggest egg cells might also synthesize DNA before fusion, this remains to be demonstrated experimentally.

Compared to male gametes, data on the DNA content of egg cells is scarce because of difficulties encountered when isolating egg cells from higher plants and because mature egg cells from some plants cannot be dyed. Mogensen and Holm (1995) measured the DNA content of isolated barley egg cells and zygotes using DAPI dye and found the DNA content of egg cells to be 1 C and zygotes, 2 C. Next, Mogensen *et al.* (1999) checked the DNA content of isolated maize egg cells and zygotes and confirmed the DNA content of egg cells at 1 C and zygotes at 2 C. They demonstrated that male and female gametes fuse at the 1 C DNA level. The male and female gametes in barley and maize contain 1 C DNA during gamete fusion, and both plants belong to the G₁ Type. Recently, Tian *et al.* (2005) examined nuclear DNA of male and female gametes of tobacco using quantitative microfluorimetry. Pollen of tobacco is bicellular and the generative cell will divide to form two sperm cells 8 h after pollination. Pollen tube growth through the 4 cm style of tobacco requires approximately two days from pollination to fertilization, and sperm cell DNA remains at 1 C (Fig. 1). When a pollen tube enters the embryo sac and discharges two sperm cells in the degenerated synergid, the two sperm cells begin to synthesize DNA (Fig. 2), and the level of DNA eventually reaches 2 C before fusion with egg and central cells. This suggests that each sperm cell starts its cell cycle and moves into the S phase after release. Concomitant with pollen tube arrival, the DNA content of the egg cell also begins to increase and finally reaches 2 C. The DNA content in newly formed zygotes is 4 C and remains at 4 C until zygote division. In the absence of pollination, the S phase in egg cells is delayed by up to 36 h, suggesting a signal reaction arising from the pollen tube (Tian *et al.* 2005). The male and female gametes

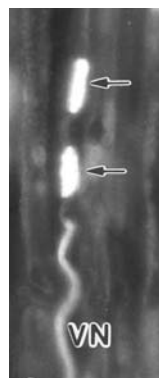


Fig. 1 Epifluorescence micrographs of vegetative nucleus (VN) and two sperm nuclei (arrows) in a tobacco pollen tube in the style. ×1500

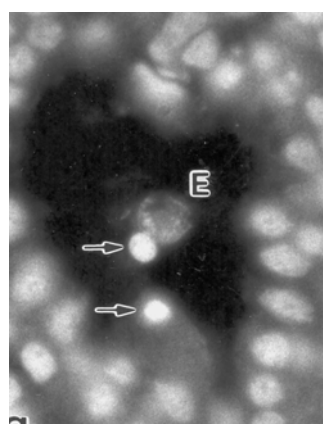


Fig. 2 Epifluorescence micrograph of two sperm cells (two arrows) in embryo sac. E: egg nucleus. ×1500

of tobacco fuse when they both finish the S phase, thus they belong to the G₂ Type.

This study not only measured the DNA content of sperm cells in pollen tubes at different stages but also measured the DNA content of mature egg cells and zygotes. The significant findings of this study were: (1) G₂ Types of male and female gametes during fertilization were confirmed in angiosperms, demonstrating the diversity of fertilization from the point of view of the cell cycle. (2) Tobacco sperm cells begin to synthesize DNA after their release and in the degenerative synergid, which is different from what occurs in *Arabidopsis* (Friedman 1999), suggesting that some mechanism in the degenerative synergid initiates DNA synthesis in tobacco sperm cells. At the same time, the initiation of DNA synthesis in tobacco sperm cells may be the final stage of development, once again indicating that tobacco sperm cells reach developmental maturation in the pollen tube or even after release in the degenerative synergid. (3) The cell cycle is a very complex signal network. Sperm cells synthesizing DNA induce egg cell activation and DNA synthesis, indicating a cell cycle signal system between both gametes. (4) Egg cells in unpollinated flowers delay DNA synthesis, meaning that egg cell DNA synthesis is itself an inherent feature but that the process can be promoted by synthesizing DNA sperm. Topics that need further study are whether DNA synthesis in egg cells is a precondition of fusion with a sperm cell and how the DNA content is changed in central cells.

Sauter *et al.* (1998) studied the expression of cell cycle regulatory genes *cdc2ZmA/B*, *Zeama;CycB1;2*, *Zeama;CycA1;1*, *Zeama;CycB2;1* and *histone H3* in sperm cells, egg cells and in other cells present in the embryo sac and the zygotes produced by *in vitro* fertilization techniques. The *cdc2* and *histone H3* genes were expressed constitutively in all cells, and the cyclin genes displayed cell-specific expression in embryo sacs and differential expression during zygote development. The expression of three cyclin genes displayed evident differences: *Zeama;cycB1;2* and *Zeama;CycB2;1* were not expressed in all cells of the embryo sac and sperm cells. *Zeama;cycA1;1* was expressed in sperm cells and all cells of the embryo sac, except in antipodals. During zygote development, three cyclin genes were expressed at different times. *Histone H3* in somatic

cells was expressed during DNA replication, but was present in sperm and egg cells, and in zygotes, for up to 24 h before decreasing in concentration. After the zygote divided, histone mRNA again appeared in the bicellular embryo.

The study of DNA contents in gametes of angiosperms has been ongoing for years. After the introduction of the concept of a cell cycle, its study in male and female gamete development has been invigorated, and many interesting new questions have arisen, a primary one being: What mechanism controls DNA synthesis in gametes and zygotes? One obvious next step for this research is to probe cyclin in both gametes and zygotes. We anticipate that the cell cycle in both male and female gametes and zygotes will shortly be an active field of study in the area of angiosperm sexual reproduction.

THE FUNCTION OF DEGENERATED SYNERGIDS

After pollen germination, pollen tubes grow in transmitting tissue and finally reach the embryo sac. The synergid cells play an important role in attracting pollen tubes and directional growth. Early studies have confirmed that calcium is involved in attracting the pollen tube *in vitro*. In tobacco embryo sacs, a difference in calcium levels between two synergids arises before synergid degeneration, and the one containing more calcium will degenerate and become the site of entry for the pollen tube. Therefore, the calcium content decides which synergid degenerates and which becomes the persistent synergid. An interaction signal mechanism appears between the pollen tube and synergid. That is, the calcium content in synergids decides the site of pollen tube entry, and the approaching pollen tube induces the change of calcium content and distribution in both synergids. It is this interaction that induces pollen tube entry at the exact location of the degenerated synergid (Tian and Russell 1997). Higashiyama *et al.* (2001) selectively killed egg cells, synergid and central cells of *Torenia fournieri* using a UV laser beam to investigate the source of attraction for the pollen tube. They found that when egg cells and the central cells were killed the pollen tube was not affected. When one synergid cell was killed the attraction frequency decreased to 71%. When two synergid cells were killed, however, no embryo sacs attracted a pollen tube, providing strong evidence that the synergid is the source of attraction.

The functions of the degenerated synergid cell are unclear. Besides for attracting the pollen tube, the arrest of pollen tube growth in time, and rupture of the pollen tube to release two sperm cells, is its another function. Usually, the tube enters the synergid from its filiform apparatus and stops elongation in the cytoplasm of synergid. Then the aperture is formed at the tube tip and the tube discharges its content into the degenerated synergid (Punwani and Gary 2008). The trigger that causes the pollen tube to form an aperture at its tip is unknown. The other functions of the degenerated synergid have not been sufficiently addressed. The special structures of the cytoplasm of degenerated synergids may function as external factors to induce tube aperture formation. A second function of the degenerated synergid may involve decomposition of the male germ unit. Two sperm cells in a pollen tube are connected with each other and associated with a vegetative nucleus constituting a male germ unit (MGU), which allows transportation of two sperm cells in the narrow space of the pollen tube (Dumas *et al.* 1994). However, the connection between the sperm cells has to be broken, allowing them to separately fuse with either the egg or the central cell. The process of breaking the connection occurs in the degenerated synergid. Very low amounts of cellulase and pectinase will remove the connection between two sperm cells (Tian and Russell 1998), suggesting that some material which removes the connection may exist in a degenerated synergid. In a third role, similarly to the above-mentioned phenomenon in which sperm cells of tobacco synthesize DNA in degenerated synergid, some mechanisms to initiate DNA synthesis in the sperm cells may exist in the degenerated synergid.

Finally, two coronas of F-actin appear at the chalazal end of the degenerated synergid and in interfaces between the egg and central cell (Huang and Russell 1994), also suggesting a physiological function after degeneration (as discussed below). Together, these data suggest that degeneration of the synergid is a developmental change related to fertilization, which plays an important physiological role in several processes during fertilization. A current view of programmed cell death can be used to explain synergid degeneration of angiosperms (Wu and Cheung 2000; An and You 2004), and could deepen our understanding of the synergid physiological function during its programmed cell death.

SIGNALS BETWEEN MALE AND FEMALE GAMETOPHYTES

There are reasons to suspect that some signals exist between male and female gametes after the pollen tube enters the embryo sac, besides interactions of pollen with stigma and pollen tube with style in incompatible pollination in some species. The distinctive events of this interaction are as follows: (1) As mentioned above, DNA synthesis in egg cells is induced by sperm cells and egg cells will delay DNA synthesis if there is no sperm cell in the degenerated synergid. (2) Degenerated synergid forms two actin coronas before pollen tubes entering it (next paragraph). (3) A pollen tube enters the degenerated synergid and discharges two sperm cells, which then induces the two polar nuclei fuse. (4) Movement of the polar nucleus or second nucleus.

Two polar nuclei of the central cell in angiosperms are relatively inactive before fertilization and cannot divide although both may fuse either before fertilization to form a secondary nucleus, or during fertilization called triple fusion. In tobacco, two polar nuclei are close to the egg apparatus and do not fuse until fertilization (Fig. 3). When the pollen tube enters the degenerated synergid and discharges its contents, including two sperm cells, two polar nuclei begin to fuse and form a second nucleus of the central cell which then fuses with a sperm cell (Fig. 4). This suggests that the sperm cell releases some signal to stimulate the fusion of the two polar nuclei prior to the fusion of three nuclei (unpublished data). In *Ranalisma rostratum*, a rare species of angiosperm, two polar nuclei are located respectively at both micropylar and chalazal ends of a mature embryo sac (Tian *et al.* 1995a). During fertilization of the central cell, a sperm cell first adheres to the micropylar polar nucleus which begins to move toward the chalazal end of the embryo sac and fuses with chalazal polar nucleus. If the flowers are emasculated and unpollinated, the micropylar polar nucleus will stay at micropylar end of central cell and not move to the chalazal end (Tian *et al.* 1995b). That sperm cell stimulates the fusion of polar nuclei in tobacco and polar nuclei moving in *R. rostratum* display sperm signals during fertilization in angiosperms. However, at the present, we know little about the signals of gametes.

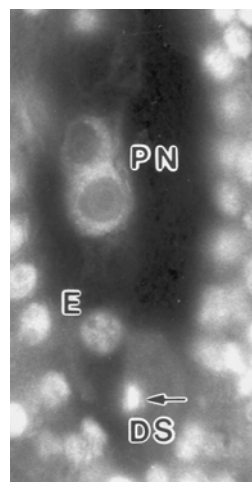


Fig. 3 Epifluorescence micrograph of a sperm cell in the micropylar end of degenerated synergid (DS). Two polar nuclei (PN) do not fuse to each other. E: egg nucleus. $\times 1200$

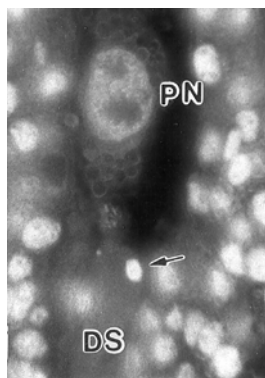


Fig. 4 Two polar nuclei (PN) fuse to each other when two sperms (arrow) move to the chalazal end of a degenerated synergid (DS) of tobacco. $\times 1200$

THE SPERM CELL TRANSPORT WITHIN THE EMBRYO SAC

In animals and lower plants, sperm cells can independently move to an egg cell. However, in higher plants, sperm cells can not move because they have no flagella. Therefore, research has focused on determining how the two sperm cells reach their target egg or central cells (**Fig. 5**). Huang *et al.* (1993) discovered that actin accumulates in the embryo sac of *Plumbago zeylanica*. In tobacco (Huang and Russell 1994), two prominent actin bands or "coronas" appear in the chalazal end of the degenerated synergid and in interfaces between the egg and central cell before the arrival of the pollen tube, and disappear before zygote division. In maize (Huang and Sheridan 1998), *Torenia fournieri* (Huang *et al.* 1999; Fu *et al.* 2000) and *Phaius tankervilleae* (Ye *et al.* 2002), the two actin coronas are also found in the degenerated synergid, suggesting that the actin coronas in degenerated synergids are not a random phenomenon.

The directional movements of organelles in cells depend on the interaction of myosin on its surface and cortical bundles of filament actin in the cytoplasm, which is very abundant in the cell with active streaming. The actin coronas in degenerated synergids consequentially have a physiological function. However, they are different from the actin filaments in cytoplasm. The actin filaments in actively streaming cytoplasm are separate and arranged in a cyclical formation. The actin coronas in degenerated synergids con-

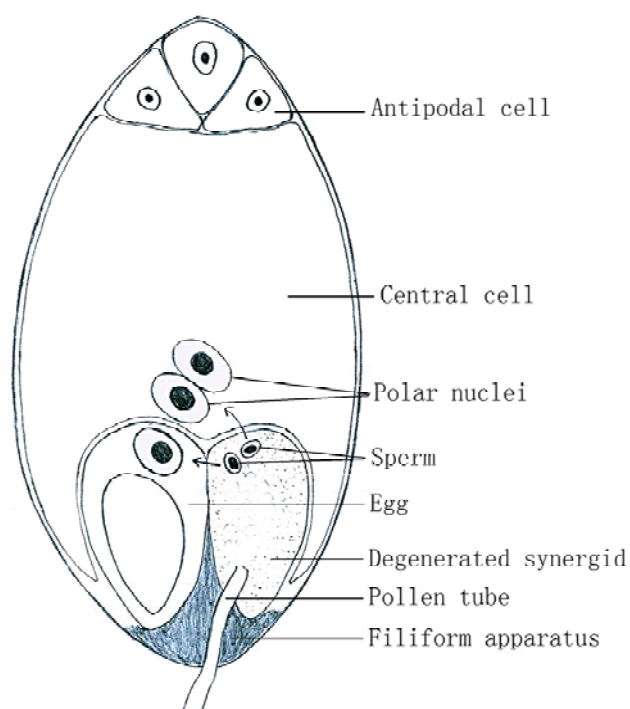


Fig. 5 A sketch of embryo sac to show two sperms orbit.

sist of many filaments accumulated in a half-moon shape which is presumably suited for one directional movement of large organelles. Huang and Russell (1994) and Huang and Sheridan (1998) presumed the actin coronas in degenerated synergids functioned as a track for both sperm cells moving to target egg and central cells.

The identification of two actin coronas in degenerated synergids is a strong support for the theory of initiative sperm cell movement in angiosperms, although both sperm cells have no flagellum. The presence of myosin on sperm cells would also support this theory. Heslop-Harrison and Heslop-Harrison (1989) found myosin on the vegetative nucleus and the generative cell in pollen grains and tubes. Zhang and Russell (1999) checked the myosin on the sperm cells isolated from pollen grains of *P. zeylanica*. Abundant myosin is located on the surface of organelles but not on the sperm. Sperm cells are negatively charged in capillary microelectrophoresis and the sperm cell that fuses with an egg has a higher calculated charge compared with the sperm cell that fuses with central cells. Myosin molecules have long, positively charged tails which may be adsorbed to the sperm cell surface due to opposite charges (Zhang and Russell 1999). There may be several explanations for why there is no myosin on sperm cells of *P. zeylanica*. First, the negative charge of sperm cells may adsorb myosin on the surface after both sperm cells are discharged. Alternatively, myosin is synthesized or adsorbed during a certain developmental phase. Because sperm cells were isolated from pollen grains and the cytoplasm is a comparatively quiet environment for sperm cells, there may be no demand for sperm cells to produce myosin for movement.

Tobacco sperm cells are positively charged in microelectrophoresis (Yang *et al.* 2005); however this is contrary to results using *P. zeylanica*. Therefore we cannot accept the hypothesis that sperm cells adsorb myosin to support active movement in the degenerated synergid. No myosin was detected on the plasma membrane of isolated tobacco sperm cells, using immunogold scanning electron microscopy. However, abundant myosin was labeled by an antimyosin antibody in the cytoplasm of pollen tubes surrounding the sperm cells (Zhang *et al.* 1999). These results suggest that myosin of pollen tube cytoplasm might help sperm cell mobility in the degenerated synergid.

PREFERENTIAL FERTILIZATION

During double fertilization, one sperm cell fuses with an egg cell and the other with the central cell (**Fig. 5**). It is unknown whether the two sperm cells fuse with their target cells by chance, or by some regulation mechanism. Russell (1984) analyzed two sperm cells in pollen grains of *P. zeylanica* using quantitative cytology and three-dimensional organization and found differences between two brother sperm cells, in volume and in organelle content, indicating dimorphism (Russell *et al.* 1996). Using information on organelle differences (mitochondria and plastids) between two sperm cells, he traced the representation of two sperm cells in fertilization and found sperm cells containing more mitochondria fusing with central cells and sperm containing plastids, with egg cells, demonstrating preferential fertilization in angiosperms (Russell 1985). The phenomenon of preferential fertilization is the recognition of male and female gametes, which has motivated many scholars to research this mechanism.

Early works about preferential fertilization have concentrated on the dimorphism of sperm cells. The dimorphism of sperm cells in some species are very evident but less so in some plants (Mogensen 1992), which makes concept of preferential fertilization suspect. Yu *et al.* (1992) studied the three-dimensional organization of two tobacco sperm cells at 8 h after pollination and compared 11 cytoplasmic parameters between both. No significant differences were found in the parameters. However, when they studied sperm cells at 26 h after pollination, the differences of volume and surface between the two sperm cells were evi-

dent (Yu and Russell 1994). Other reports also confirmed that the volume difference between tobacco sperm cells increased with development (Tian *et al.* 2001). Therefore, if a pair of sperm cells do not display dimorphism in some plants, there may be two possible explanations. The time of sampling might have been too early to observe the difference, or the differences between the two sperm cells were too small to detect visually. In *P. zeylanica*, the surface charges also differ between the two sperm cells. The sperm cell that normally fertilizes the egg has a higher charge (Zhang and Russell 1999). Whether the differences between two sperm cells can be seen or be measured, the incidence of double fertilization, in that two sperm cells from one pollen tube fuse individually with either the egg or central cell, is itself evidence of selective fertilization. Otherwise, we would also occasionally observe the two sperm cells fusing with egg or central cells synchronously.

Molecular-level study of sperm cells has become an active field in gametic recognition following sperm cell isolation from many plants. Studies on animals and lower plants have shown that the surface glucoproteins of egg and sperm cells play an important role on gametic recognition during fertilization. Southworth and Kwiatkowski (1996) tested sperm cells of *Brassica* using the antibodies of arabinogalactan proteins and found two monoclonal antibodies, JIM8 and JIM13 that bind to the surface of sperm. They proposed that the antibodies of arabinogalactan proteins may be useful for identifying sperm from other cell types. Xu and Tsao (1997a) used six lectins to label maize sperm cells and found RCA 1, Con A and WGA can be labeled, suggesting galactose, mannose, glucose, and N-acetylglucosamine residues on the plasma membrane of maize sperm cells. They also isolated several glucoproteins from plasma membranes of sperm cells (Xu and Tsao 1997b). These glucoproteins may include some lectin receptors. However, the function of these glucoproteins in the recognition of gametes requires further study.

Additional research on gametic recognition led to the isolation of special genes which control the recognition of male and female gametes. Xu *et al.* (1999a) isolated and characterized two cDNA clones encoding generative cell-specific histones, gCH2A and gCH3, from *Lilium longiflorum*. They further isolated a male gametic cell-specific gene, LGC1, and confirmed its expression. The gene product was located on the surface of male gametic cells, and the product may be involved in the interactions between male and female gametes (Xu *et al.* 1999b). Ueda *et al.* (2000) isolated three genes encoding three histone proteins from *L. longiflorum*. The proteins were present in both sperm cells suggesting that they might be involved in condensing chromatin of male gametes or perhaps inhibiting gene expression of male gametes. Xu *et al.* (2002) constructed a cDNA library of mRNA isolated from pure tobacco sperm cells, and distinguished 2 cDNAs, NtS1 and NtS2, representing sperm cell expressed transcripts from an initial screen of 396 clones. NtS1 codes for a polygalacturonase, suggesting a role for this enzyme in wall degradation during differentiation of sperm cells in tobacco. Singh *et al.* (2002) isolated polyubiquitin-encoding cDNA clones from the generative cells of *L. longiflorum* and the sperm cells of *P. zeylanica*. They proposed that the gene participates in ubiquitination of proteins and may enhance protein turnover in the germline during male reproductive differentiation. Recently, Russell's lab made two cDNA libraries of *P. zeylanica* based on the isolation of two populations of sperm cells (Gou *et al.* 2004). This is a first attempt at screening out the special genes between two sperm cells and made a basis for preferential fertilization. For distinguishing between two sperm cells, over a thousand individual populations of the two dimorphic sperm cells were isolated from pollen tubes of tobacco (Yang *et al.* 2005) and *T. foeneri* (Chen *et al.* 2006). The collection of these two individual populations containing over a thousand sperm cells will permit synthesis of cDNA in sperm cells for use in researching gametic recognition of the plants with bicellular pollen.

Preferential fertilization by two sperm cells from one pollen tube was reported in 1985 in *P. zeylanica* (Russell 1985); however, the phenomenon has not been reported in other species to date, probably due to technological difficulties. Plants having different organelles in two sperm cells and having critical positions at which sperm adhere to egg cells, to distinguish the sperm cells, need to be found. It is very difficult to satisfy this condition using electron microscope techniques. Initially, in *Phaius tankervillea*, both sperm cells stay close together in the degenerated synergid. Then the leading sperm cell migrates towards the central cell, while the other sperm cell moves laterally towards the egg cell (Ye 2002). This action suggests the occurrence of preferential fertilization in this plant but identification of the differences between the two sperm cells is required to complete our understanding of this phenomenon.

EGG CELL ACTIVATION

Studies on animals and lower plants have shown that a calcium change is a prime signal action within cells (Stricker 1999; Ciapa and Chiri 2000). In a previous review, it was pointed out that in higher plants, the fusion of sperm cells with an egg cell triggered a transient cytosolic Ca^{2+} increase in the fertilized egg cell (Wang *et al.* 2006). After 29 min, the calcium level returned to the same level as was observed in the unfertilized egg cell (Digonnet *et al.* 1997). Based on their observations, Antoine *et al.* (2000) proposed a process of initial fertilization where (1) membrane fusion of both gametes starts a calcium influx close to the fusion site; (2) a wave of contraction, presumably of actomyosin nature, is triggered; and (3) this in turn, triggers the opening of more stretch-activated calcium channels. When the Ca^{2+} channels are inhibited by Gd^{3+} , the fusion of sperm with an egg cell still induces a cytoplasmic calcium increase, and presumably calcium is released from intracellular stores (Antoine *et al.* 2001a). The rise of calcium contributes to a calcium signal and triggers egg cell activation. Although calcium-triggered egg cell activation is very interesting, several questions need answering: Does the sperm cell fuse with an egg cell in a special site, or target site *in vivo*, is the fusion site of *in vitro* fertilization found by chance, and is there any difference of fusion site between *in vivo* and *in vitro* fertilization? Another interesting question is whether parthenogenesis will occur if ionophore A23187 is used to increase calcium in the egg cell or IP_3 is used to release Ca^{2+} from the calcium stores of egg cells.

In their work based on the hypothesis that calcium triggers egg cell activation by a transient cytosolic Ca^{2+} increase, Tirlapur *et al.* (1995) observed an increase in cytoplasmic calmodulin (CaM) levels in artificial zygotes compared to isolated egg cells, suggesting that Ca^{2+} may trigger egg cell activation by the CaM pathway. Therefore, we propose that the natural next step is to explore the action induced by calcium elevation.

Up until now, data on egg cell activation in angiosperms has been obtained by *in vitro* fertilization only in maize, *Zea mays* inbred line A188 (Kranz and Lörz 1993). The existing results have left several unanswered problems. Prior to fertilization, egg cells have some interactions with contiguous cells (synergid and central cells) and surrounding somatic tissue, but during *in vitro* fertilization only sperm and egg cells fuse without these interactions. The sperm cells used in maize for *in vitro* fertilization was from pollen grains and did not develop from a pollen tube growing in a style. It is unknown how these two factors will affect egg activation. There is also the question of the difference in how egg cell activation might be triggered between two maize sperm cells from one pollen tube. Finally, is the phenomenon of egg cell activation in maize representative of all angiosperms? We consider it essential to confirm this phenomenon in other plants, especially in dicotyledons.

CELLULAR DETERMINATION OF ZYGOTE

Since the zygote is the first cell in the ontogeny of a plant, understanding its development is a very interesting research topic. Following a predetermined mode of development it gives rise to an embryo with the potentiality to form a complete plant. Generally, most zygotes of angiosperms divide asymmetrically to form a bicellular proembryo consisting of a large basal cell and a small apical cell. In many plants the basal cell of bicellular proembryo divides to form a suspensor or does not divide and plays only a minor role or none in the subsequent development of the proembryo, and the apical cell develops an embryo-young plantlet. What makes so large difference between two daughter cells? We can deduce that the polarity of the egg cell is a reason of asymmetrical division of zygote. But why the fate of two cells of bicellular proembryo is so different in late development? In another words, what is the mechanism of the zygote development? Recently, Okamoto *et al.* (2005) identify some genes that were up- or down-regulated only in the apical or basal cell of maize bicellular proembryo using *in vitro* fertilization. They synthesized cDNAs from apical cells, basal cells, egg cells bicellular proembryos and multicellular embryo, and used these cDNA as templates for polymerase chain reaction (PCR) with randomly amplified polymorphic DNA (RAPD) primers. Genes with specific expression patterns were categorized into six groups. They found that genes up-regulated in apical or basal cell after fertilization were revealed to be expressed in the early zygote. For this result, they deduced three possibilities: (1) transcripts from these genes are localized to the putative apical or basal region of the zygote; or (2) the transcripts from these genes may exist uniformly in the egg cells and become distributed to the apical and basal regions of zygote after fertilization; or (3) the transcripts might be degraded in one of daughter cells immediately after zygotic cell division. They prepare to conduct visualizing the putative subcellular location of mRNA in maize zygote using microinjection of fluorescence-labeled RNA into zygotes or *in situ* hybridization methods (Okamoto and Kranz 2005). Ning *et al.* (2006) also found that (1) clusters of genes present in egg cells persistent in zygotes, (2) clusters of genes represented in sperm cells and persistent in zygotes, as well as (3) a large cluster of genes produced by apparently new transcripts within the zygote. Their report includes one histone gene present in both the egg cell and zygote, which may represent one potential mechanism by which gene networks are controlled. Although the reports about cellular determination of zygote are a few, these excellent results point the way for exploring the mechanisms of cellular determination during early embryogenesis in higher plants.

CONCLUSIONS AND PROSPECTS

Fertilization in angiosperms is a complex and accurate process, and has many interactions between male and female gametes. The ultrastructural studies of generative cells using electron microscopy since 1970's enhanced our understanding of structural and physiological functions of generative cells. Now research on the reproductive biology of angiosperms has entered a new phase where methods of molecular biology will permit many questions about the fertilization mechanism to be answered. There are different recognitions during sexual reproduction of angiosperms: sporophytic recognition occurs between pollen and stigma and style; gametophytic between pollen tube and degenerated synergid; gametic between sperm and egg cell. All of the recognitions actually are interactions between male and female cells, especially when pollen tube approaching embryo sac, egg and central cells take place some morphological and physiological changes, suggesting a signal network in the process. There are still many questions of fertilization mechanism occurring in ovule needing to answer. In above-discussed topics cell cycle of gametes is reported only in *Arabidopsis* and tobacco, need to check more plants.

Using the point of view of program cell death to study the old problem of synergid degeneration will produce some new results. The sperm movement in degenerated synergid is an interesting problem. Although the actin coronas in degenerated synergid have been found in several plants, the movement of two sperm cells in synergid still needs to prove from the myosin distribution on sperm cells and the manner of interaction between myosin and actin, which may differ from organelles movement in cytoplasm. Since preferential fertilization by two sperm cells from one pollen tube was reported in *Plumbago zeylanica* in 1985, the phenomenon was not reported until now and needs to be proved in more plants. We have found some phenomena of recognition between male and female gametes in angiosperms, but we know little about these recognition mechanisms which without doubt are core projects of fertilization study. Using of references of the methods and thoughts in animals and lower plants, the studies of egg activation and cellular determination of zygote of angiosperms were conducted and revealed excellent results using *in vitro* fertilization. The outcome of these researches will finally provide answers to many of the important questions about the process of double fertilization.

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