

REVIEW

Synergid: a key link in fertilization of angiospermsD.X. LI¹, M.Z. LIN², Y.Y. WANG³ and H.Q. TIAN^{1*}*School of Life Sciences, Xiamen University, Xiamen, 361005, P.R. China¹**Zhangzhou Medical Vocational College, Zhangzhou, 363000, P.R. China²**Xiamen Medical College, Xiamen, 361008, P.R. China³***Abstract**

In over 80 % of the angiosperms, the female gametophyte is comprised of seven cells, two of which are the synergid cells. These cells are considered pivotal in assuring successful fertilization. The synergid cells direct pollen tube growth toward the female gametophyte, and facilitate the entrance of the tube into the embryo sac. Once the pollen tube enters the synergid cell, its growth is arrested, the tip of the tube breaks, and two sperm cells are released. This sequence of events is also synergid dependent. In addition, separation of the cells of the male germ unit, orientation of the two sperm cells in the degenerating synergid, and fusion of the egg and central cell with sperm cells may also be related to synergid cells. Synergid structure has been widely studied, but development and function of these cells during angiosperm fertilization remains elusive. Recent molecular approaches have provided an enhanced understanding of the role of synergid cells in fertilization. The present review summarizes the results of current studies regarding the role of synergids in angiosperm reproductive function.

Additional key words: development, embryo sac, fertilization, synergid function.

Introduction

The two synergid cells characteristic of most angiosperm embryo sacs are specialized to assist in fertilization. The cells are haploid components of the female gametophyte located beside the egg cell (Fig. 1). Following pollination, the pollen tube enters an ovule through the micropyle, and then proceeds to enter a degenerated synergid. The growth of the pollen tube is arrested in the synergid and the tip breaks to release two sperm cells into the degenerated synergid. This process initiates fertilization. One sperm cell migrates toward the egg, and the other towards the central cell of the embryo sac. The fusion of one male gamete and female gamete results in the formation of a diploid zygote. The often nearly simultaneous fusion of second male gamete and the central cell leads to the development of a typically triploid endosperm. This sequence of events completes double fertilization, characteristic of most angiosperms (Lord and Russell 2002, Weterings and Russell 2004). Only one of the mature synergids receives the pollen tube prior to entering the female gamete. Structural observations of synergid cells have been well described and the main physiological function attributed to the synergids is to attract pollen tube

to the embryo sac (Willemse and Van Went 1984, Huang and Russell 1992). Published review papers on the study of synergids are numerous (Willemse and Van Went 1984, Huang and Russell 1992, Drews *et al.* 1998, Drews and Yadegari 2002, Higashiyama 2002, Yadegari and Drews 2004), indicating an interest in the role of synergids in plant reproductive biology. In a recent review on synergid development and function, Punwani and Drews (2008) discussed the function of synergids in attracting pollen tubes. The most recent studies have employed genetics, molecular biology and physiological methods. However, little research is available addressing synergid developmental mechanisms. It has been demonstrated that the two synergid cells originate from one nucleus located in the micropylar end of a four nucleate embryo sac. However, why the structure and function of two synergid cells are so different, each one flanking the egg cell, remains elusive.

The aim of present paper is to survey results from former and recent research on the reproductive functions of synergids.

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* Corresponding author; e-mail: hqtian@xmu.edu.cn

Structure of the female gametophyte

Synergid cells are formed during female gametogenesis. Over 80 % of all embryo sacs identified in angiosperms are the *Polygonum* type. This type of embryo sac is characterized by meiotic production of four megaspores from the megasporocyte. However, three megaspores degenerate and only the chalazal megaspore continues to develop. The functional megaspore subsequently undergoes three mitotic divisions to form an eight nuclear embryo sac. A cell wall subsequently develops between nuclei and forms a female gametophyte that contains seven cells and eight nuclei. Four cell types are recognized, including one egg, two synergid cells located at the micropylar end flanking the egg, three antipodal cells at the chalazal end, and a large central cell with two polar nuclei situated in the middle of the cell (Huang and Russell 1992; Russell 1993). Thus the sac cytoplasm determines the fate of the eight nuclei, or alternatively the differentiation of these nuclei is dictated by their position in the sac (Russell 1993, Christensen *et al.* 1997, Drews and Yadegari 2002). Davidson *et al.* (1998) suggested that

the nuclear differentiation of the female gametophyte is determined by an asymmetry in the 4-nuclear embryo sac. During the division of two micropylar nuclei in the 4-nuclear embryo sac of *Lycium barbarum* (*Solanaceae*), one spindle is vertical to the embryo sac ordinate axis, and the two newly formed nuclei are symmetrical, which form two synergid nuclei. The other spindle is parallel to the embryo sac ordinate axis, the division is asymmetrical, which forms two different nuclei: the egg nucleus and a micropylar polar nucleus in the large central cell (Tian 1987; Fig. 1a). In most plants, the embryo sacs are entirely enclosed within the ovule, which leads to difficulties in directly observing the embryo sac. However, the embryo sac of *Torenia fournieri* (*Scrophulariaceae*) partially protrudes through the micropyle. Thus, the egg cell, two synergid cells, and part of the central cell can readily be observed using light microscopy. These features of *T. fournieri* make it an attractive model to investigate synergid function (Higashiyama 2002).

Synergid ultrastructure

At maturity, both synergids develop special structures, including an uneven thickening. For both synergids the thinning, and absence or loss of the chalazal end wall are also typical. Some synergids form a large vacuole at the chalazal end, which displaces its nucleus and most of the cytoplasm toward the micropylar end. This leads to a reversed polarity with respect to that of the egg cell. Presence of abundant ribosomes, mitochondria, Golgi bodies, and endoplasmic reticulum in synergid cytoplasm suggest that synergids are biochemically active and dynamic cells (Willemse and Van Went 1984, Huang and Russell 1992). The most obvious feature of synergids is an uneven cell wall, which is frequently discontinuous at its chalazal end, which facilitates the passage of sperm nuclei into female cells and their subsequent migration toward the egg and polar nuclei (Willemse and Van Went 1984). The wall at the micropylar end is thickened, and forms many fingerlike projections into the cytoplasm, called the filiform apparatus. Staining shows the filiform apparatus is composed of cellulose, hemi-cellulose, pectin and proteins. Structurally, the filiform apparatus exhibits abundant secretory organelles, such as endoplasmic reticulum, Golgi bodies, and vacuoles. Therefore, it has been proposed that the filiform apparatus facilitates the movement of materials into and out of the synergids. Ultrastructural observations indicate the pollen tube always enters the synergid from the filiform apparatus, so it can be considered a receptor for transfer of the pollen tube to the female gametophyte (Willemse and Van Went 1984, Huang and Russell 1992).

Kasahara *et al.* (2005) isolated a gene controlling some aspects of synergid development. The *MYB98* gene, a member of the R2R3MYR gene family, was isolated from

Arabidopsis thaliana. This gene encodes transcription factors associated with the *MYB98* gene. The *myb98* mutant displayed an abnormal filiform apparatus at the micropylar end of the synergid, exhibiting an unusually homogeneous wall distribution. The mutant lost the ability to attract the pollen tube to the embryo sac. Therefore, *MYB98* is necessary for synergid differentiation (Kasahara *et al.* 2005). Recently, Punwani *et al.* (2007) further tested the predicted function of *MYB98* as a transcriptional regulator with a subcellular localization and its DNA binding properties. The study found that *MYB98* protein integrated a specific sequence of DNA (TAAC), and a *MYB98*-green fluorescent fusion protein was localized to the nucleus. The study tested 16 previously identified synergid-expressed genes that showed lower expression in *myb98* female gametophytes. Results confirmed that one of the genes, *DD11*, was regulated directly by *MYB98*. Five proteins transcribed by an additional five downstream genes were secreted into the filiform apparatus. They suggested that *MYB98* functions as a transcriptional regulator in synergid cells, and activates the expression of genes required for filiform apparatus formation (Punwani *et al.* 2007). An *Arabidopsis* mutant (*lachesis*, *lis*) was developed where accessory cells serve as egg cells (*e.g.* accessory cells differentiate into gametic cells in the embryo sac), and the change in function of these cells is dictated by a defect in the gametic cells (Gross-Hardt *et al.* 2007). The *LIS* gene is involved in preventing accessory cells from becoming female gametes. *LIS* is homologous to the yeast splicing factor PRP4, suggesting that components of the splicing apparatus participate in cell fate decisions (Gross-Hardt *et al.* 2007).

Most angiosperms possess two synergids in the

embryo sac. However, during fertilization, one normally degenerates and receives the pollen tube, whereas the other is persistent. An interesting question is whether the pollen tube enters a synergid by chance or by selection? In mature embryo sacs of *Allium tuberosum* (Liliaceae), the two synergids displayed distinct size differences. However, this result was obtained by section, resulting in a two-dimensional depiction. The result has not been confirmed by additional lines of evidence (Tian and Yang 1991). Recently, the size difference between two synergids was described by the isolation of the egg apparatus in *Brugmansia aurea* (Solanaceae). At day 3 after anthesis, the two synergids were of the same size. However, size difference between the two synergids

appeared 6 d after anthesis (Fig. 1b-d; unpublished data). At anthesis in *Allium fistulosum*, the two synergids were of the same size, but at day 3 following anthesis, one synergid was over 35 % larger than the other (Fig. 1e,f; unpublished data). Presently, the factors responsible for size differences between the two synergids are uncertain, but may be related to pollen tube reception. In tobacco, prior to the structural changes leading to a degenerated synergid, the cell displayed increased amount of calcium precipitates relative to the second synergid cell. The synergid containing more calcium degenerated when the pollen tube arrived, which supports functional differences between the two synergids (Tian and Russell 1997).

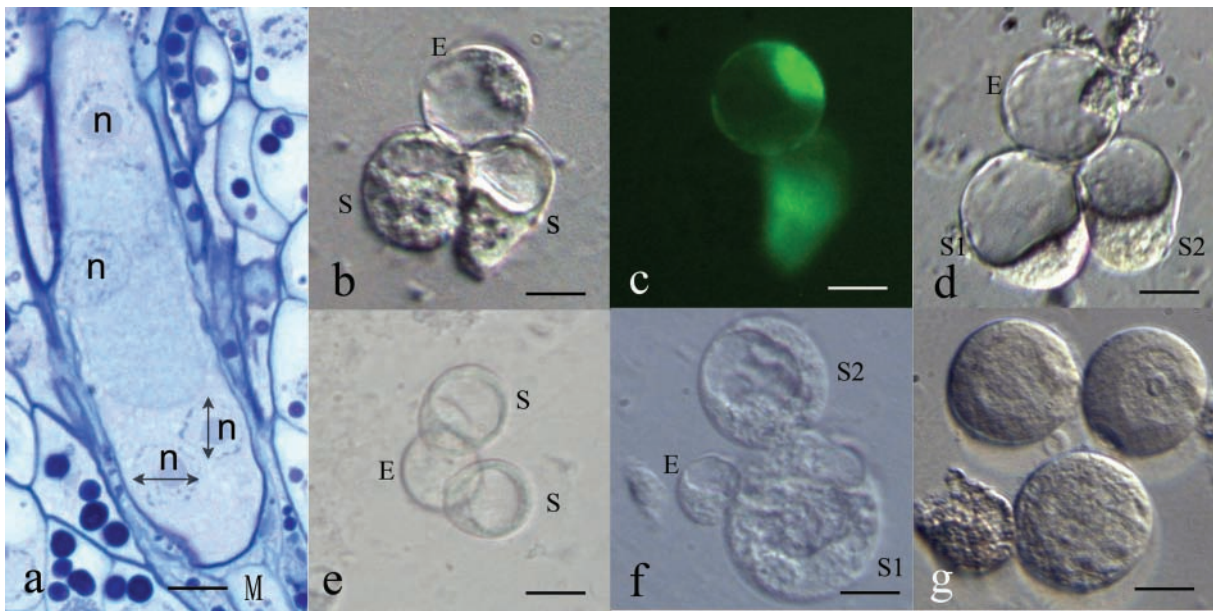


Fig. 1. *a* - a sketch of embryo sac to show two synergids and filiform apparatus; four nuclear embryo sac of *Pseudostellaria heterophylla* to show the division direction of two nuclei in micropylar end of an embryo sac; *b* - three cells of egg apparatus of *Brugmansia aurea* isolated 3 d after anthesis. *c* - epifluorescence micrograph displaying the viability difference between two synergids evaluated by FDA reaction; *d* - egg apparatus of *Brugmansia aurea* isolated 6 d after anthesis; *e* - the three cells of isolated egg apparatus of *Allium fistulosum* at anthesis; *f* - the same 2 d after anthesis, showing cell size difference between two synergids; *g* - three isolated synergids of *Allium tuberosum*. E - egg cell, S - synergid. Bar = 10 μ m.

Synergid functions

Guiding pollen tube: Following pollination the pollen tube enters the ovule through the micropylar region. Initially, the role of synergids in pollen tube orientation through the micropyle was not well understood (Van Went and Willemse 1984). Shimizu and Okada (2000) reported that the ovule of *Arabidopsis* is anatropous, and its micropyle lies adjacent to the funiculus. In the mutant *maal* from *Arabidopsis*, the ovules lost the capacity to attract the pollen tube. In yet another mutant *maa3*, the pollen tubes could reach the funiculus, but not enter the micropyle. Results of the study suggested female gametes produced two guide signals for pollen tubes. One induced pollen tube growth from the placenta to the funiculus, and

the other induced pollen tube growth from the funiculus to the micropyle (Shimizu and Okada 2000). Tobacco also bears an anatropous ovule, and once in the placenta, the growing pollen tubes veer at a right angle to reach the micropyle. Calcium distribution studies in tobacco ovules indicated that the micropyle entrance and synergid filiform apparatus accumulated the highest amount of Ca precipitates. It was hypothesized that high content of Ca precipitates at the micropyle entrance guided the pollen tube to the ovule, and Ca in the filiform apparatus assisted the pollen tube in reaching the embryo sac (Tian and Russell 1997). The micropyle of *Plumbago zeylanica* (Plumbaginaceae) also accumulated abundant Ca precipi-

tates, again suggesting that high Ca deposition in the micropyle guided the pollen tube to the ovule. *P. zeylanica* lacks synergid cells, and abundant Ca precipitates are located in the egg cell wall. These data further supported that Ca aids the ovule in attracting and receiving the pollen tube to achieve fertilization (Tian *et al.* 2000). In addition, the micropylar cells of pearl millet and *Brassica napus* exhibited abundant Ca deposition (Chaubal and Reger 1992, Yu *et al.* 1998).

An understanding of synergid cells is not complete without elucidating the development and function of the filiform apparatus. The fingerlike extensions of the apparatus increase the surface area of the plasma membrane, and may serve in both absorbance and secretion functions. The primary biological function of synergids is to attract pollen tubes. *In vitro* studies of *Antirrhinum majus* revealed the pollen tubes display a chemotropic response to external Ca gradients (Mascarenhas and Machlis 1962). The synergid filiform apparatus maintains the highest amount of Ca precipitates. After a pollen tube passes through the filiform apparatus, it stops growth and the tip breaks to release the tube contents, including two sperm cells. Therefore, the high Ca content in the synergid filiform apparatus appears to be related to attracting the pollen tube. However, the precise mechanism regulating this process is unknown. According to the characteristics of the filiform apparatus, we suggest the following: the filiform apparatus enlarges the plasma membrane surface, which can rapidly secrete Ca ions in a short time; the filiform apparatus thickens, which can subsequently accumulate abundant Ca ions. It is a structural feature of the filiform apparatus to attract the pollen tube. In addition, accumulation of Ca in the cell wall may prevent a cytoplasm from toxic effect of high Ca content in both synergid cells.

Studies on *Torenia fournieri* provided direct confirmation of the role of synergids in attracting the pollen tube. Light microscopy shows that the mature embryo sac with its egg cell, two synergids and part of the central cell protruded partially through the micropyle. Higashiyama *et al.* (2001) used a laser to destroy the cells of the egg apparatus, and further confirmed that the synergids attract the pollen tube. They co-cultured the ovules and pollen tubes together, and the tube entered the embryo sac. When the egg cell was destroyed, the pollen tube still entered the embryo sac, however, when one synergid was eliminated, the pollen tube entered another synergid and when both synergids were destroyed, the pollen tube did not enter the embryo sac. These results more definitively established the role of synergids in fertilization. In addition, the study also found that following fertilization, a pollen tube would not enter the persistent synergid, indicating the synergid cell prevents additional pollen tubes from entering the embryo sac.

Recently, several genes related to synergid function have been identified. *ZmEAL* was exclusively expressed in the egg apparatus cells of maize, and the gene encoded a small 94 amino acid maize protein, required for pollen tube attraction by the female gametophyte. Chimeric

ZmEAL fused to a green fluorescent protein (*ZmEAL:GFP*) was firstly visible within the filiform apparatus. The protein was later localized to nucellar cell walls below the micropylar opening, suggesting *ZmEAL* was secreted by synergid cells. Transgenic down-regulation of the *ZmEAL* gene led to ovule sterility caused by the loss of close-range pollen tube guidance to the micropyle (Marton *et al.* 2005). Kasahara *et al.* (2005) identified *MYB98* in a screen for *Arabidopsis thaliana* genes expressed in the female gametophyte. *MYB98* was expressed exclusively in synergid cells, and mutations in this gene affected the female gametophyte resulted in a loss of pollen tube attraction to the ovule. These results suggested that *MYB98* controls the development of specific features within the synergid cell during female gametophyte development (Kasahara *et al.* 2005). The loss of pollen tube attraction in the *myb98* mutants lacking a normal filiform apparatus may have another explanation and Kasahara *et al.* (2005) did not address changes in micropyle function in attracting the pollen tube. The pollen tube entrance into the ovule and embryo sac is comprised of two distinct processes; one process is closely related to the micropyle, and the other to synergids. Calcium distribution and content in the *MYB98* mutant micropyle would likely provide more insight into *MYB98* function. Recently, Rotman *et al.* (2008) reported a new mutant *scylla* (*syl*) in *Arabidopsis thaliana* with impaired control of pollen tube discharge. The study suggested that pollen tube discharge was controlled by an interaction between the synergids, expressing the *SRN/FER* gene and the central cell expressing *FIS* genes. The molecular characteristics of synergid will become a new focus of plant reproductive biology following a quantity of synergids isolated (Fig. 1g, unpublished data).

Induction of synergid degeneration: Ultrastructural observations indicate that the synergid receiving the pollen tube follows a cell death process resulting in a degenerated synergid, which provides a gateway for the entrance of sperm into the egg and central cells (Van Went and Willemse 1984, Russell 1993, 1996). The pollen tube only enters the degenerated synergid. Therefore, synergid breakdown is essential in receiving the pollen tube. In some plants, a synergid degenerates at anthesis, without pollination, but in others, the synergid degenerates only after the approaching pollen tube. Three types of synergid degeneration have been described in angiosperms: 1) the synergid degenerates before pollination, due to developmental processes not related to the pollen tube; 2) synergid degeneration occurs before the arrival of the pollen tube following pollination, suggesting synergid degeneration is related to the pollen tube signal (most angiosperms are of this type) (Russell 1992, Drews and Yadegari 2002); and 3) degeneration is initiated when the pollen tube enters the synergid cell and is induced by direct contact between the pollen tube and the synergid (Russell 1992, Sandaklie-Nikolova *et al.* 2007). Only a few plants exhibit this type. Interestingly, in the second type of synergid degeneration, a recognition mechanism between male and female

gametophytes has been reported. In tobacco, synergid degeneration occurred as the pollen tube arrived at the ovary, but not the embryo sac. If pollination was prevented, or the style was cut off to prevent migration of the pollen tube to the ovule, the synergid would not degenerate. These observations suggest that the tube in fact induces the degeneration of a synergid, and the effect acts shortly after the tube reaches the ovary (Huang and Russell 1992). If the flower is emasculated, both synergids remain intact until the flower senesces and drops 6 d after anthesis (Tian and Russell 1997). A signal transduction even generated by interactions between the pollen tube and the synergid, stimulate high Ca accumulation in the apparatus filiform, which facilitates pollen tube entry into the embryo sac. The arrival of the tube in the ovule initiates synergid degeneration. In addition to tobacco, many angiosperms exhibit these same phenomena, indicating this may be a universal feature of flowering plants.

The angiosperms are a highly diverse group of plants, and synergid degeneration processes exhibit variability consistent with angiosperm diversity. Synergid degeneration is typical of the type of programmed cell death (PCD) observed in many different organisms. In *Arabidopsis*, several genes are involved in synergid PCD. In the mutant *gfa2*, synergid degeneration did not occur after the pollen tube entered the female gametophyte. *GFA2* encodes a mitochondrial protein, suggesting synergid death is related to mitochondrial function (Christensen *et al.* 2002). In the mutant *srn*, similar to *gfa2*, synergid degeneration did not occur following pollen tube entry into the female gametophyte (Rotman *et al.* 2003). In the mutant *feronia* (*fer*), when fertilization was impaired, pollen tube migration failed to cease, and the tube continued to grow in the female gametophyte. *FER* encodes a synergid-expressed, plasma membrane-localized receptor-like kinase, which accumulated asymmetrically in the synergid membrane at the filiform apparatus (Escobar-Restrepo *et al.* 2007). The results of these studies support the pollen tube induced PCD of synergid cells.

Pollen tube growth regulation in synergid cells: After the pollen tube penetrates the filiform apparatus, growth of the tube is arrested inside the degenerated synergid. The tube tip subsequently breaks and releases two sperm cells into the cytoplasm of the degenerated synergid. The mechanisms involved in the cessation of tube growth, including the breaking of the tube tip and tube content release are not fully understood. In *Arabidopsis* mutants *fer* (Huck *et al.* 2003) and *srn* (Rotman *et al.* 2003), pollen grains of the wild type germinated on the mutant stigma, and the pollen tube grew into the mutant synergid, but growth was not arrested and the tube tip did not break. This suggested the pollen tube was under the control of the female gamete. Pollen of interspecific crosses using *Arabidopsis lyrata* and *Cardamine flexuosa* (*Brassicaceae*) on *A. thaliana* stigmas behaved similarly as a *fer*-like type, but the pollen tube continued to grow through the synergid and into the female gametophyte. It was subsequently determined that female control of pollen tube reception

was based on a FER-dependent signaling pathway (Escobar-Restrepo *et al.* 2007). Alternatively, the degenerated synergid may control pollen tube growth because growth is arrested in a degenerated synergid (Van Went and Willemse 1984, Russell 1992, Weterings and Russell 2004). Yet another plausible explanation exists. The synergid filiform apparatus contains a high Ca content in comparison with the cytoplasm (Tian and Russell 1997). Therefore, after the pollen tube passes through the filiform apparatus, the cytoplasm is not as suitable for tube growth and growth is discontinued.

Other synergid functions: Synergid structure is indistinct following degeneration, and synergid function after pollen tube entry has received little attention. However, in addition to pollen tube attraction, cessation of pollen tube growth, and pollen tube tip rupture, other biological events prior to fertilization have been reported in synergids. As previously discussed, the tube tip breaks and discharges its contents into the degenerated synergid. However, the point of rupture is at a terminal aperture. The trigger that causes aperture formation is unknown. During the isolation of sperm cells in tobacco, it was suggested that a form of osmotic shock in the synergid resulted in the dissociation of the pollen tube. However, when cultured pollen tubes were transferred from a 15 % sucrose to a 9 % mannitol solution, only some tubes burst (Tian *et al.* 1998). Therefore, specialized cytoplasmic components in the degenerated synergid may function as external factors to induce tube aperture formation.

A second biological function involves decomposition of the male germ unit. The two sperm cells in a pollen tube are connected to each other and associated with a vegetative nucleus in an assemblage known as the male germ unit (MGU). The MGU allows the transport of the two sperm cells in the narrow space of the pollen tube (Dumas *et al.* 1994). However, the connection between the sperm cells must be broken, allowing them to separately fuse with either the egg or the central cell, and this connection seems to be broken in the degenerated synergid. In isolated tobacco sperms, low amounts of cellulase and pectinase could also remove the connection (Tian and Russell 1998, Yang *et al.* 2005). Presumably similar enzymes might be present in a degenerated synergid. Huang and Russell (1994) found that two prominent actin coronas appear at the chalazal end of the degenerated synergid and are associated with the pathway of the sperm cells toward the egg and central cell of tobacco. In *T. foeneri* (Huang *et al.* 1999) and in *Phaius tankervilleae* (*Orchidaceae*) (Ye *et al.* 2002), also found two actin coronas in degenerated synergids. One function of cellular actin is to facilitate transport by interaction of myosin on the organelle surface with filament actin in the cytoplasm. Huang and Russell (1994) and Huang and Sheridan (1994) presumed that actin coronas in degenerated synergids functioned as a track for both sperm cells during their migration to the egg cell and central cell. These results further support synergid degeneration as a physiological transformation to ensure successful fertilization.

Generally, the DNA content of male and female gametes of higher plants is at 1 C of relative content. However, Friedman (1999) found that the sperm cells of *Arabidopsis thaliana* begin to synthesis DNA in the pollen grain and both reach nearly 2 C prior to fusion with the egg cell. In tobacco, Tian *et al.* (2005) examined nuclear DNA of male and female gametes using quantitative microfluorimetry. 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, 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 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, when fusion occurs. The DNA content in newly formed zygotes is 4 C and remains at 4 C until zygote division (Tian *et al.* 2005). The initiation of the cell cycle of both sperm cells is another function proposed for synergids. Mechanisms to initiate DNA synthesis in the two sperm cells may be regulated by the degenerated synergid (Hu *et al.* 2008).

Conclusions

The synergids are an important component of the female gametophyte in angiosperms and control several key processes to ensure successful fertilization. The structure of synergids and the filiform apparatus are unique. Many synergid functions have been recognized, including the attraction of the pollen tube, the arrest of pollen tube elongation in the degenerating synergid, and rupture of the pollen tube to release the tube contents. The control mechanisms have been studied using a variety of

approaches, including molecular screening and light microscopy. Although other roles of degenerated synergids have been recognized, for example the mechanisms responsible for the movement of sperm cells in the synergid require further study. Recently, genes involved in synergid function have been identified in *Arabidopsis* mutants that have contributed to our knowledge of their role of synergid cells.

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