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Exploring the sister cells of embryo sac: developmental and functional attributes

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Abstract

Synergids are metabolically dynamic cells of the egg apparatus and represent an important component of the female gametophyte. Besides directing the growth of the pollen tube towards the micropylar end of the embryo sac, these ephemeral structures make room for the pollen tube cytoplasm. The nature of chemotrophic substances that direct the growth of the pollen tube, the mechanism of degeneration of one of the synergids before fertilization and the molecular aspects of synergid morphogenesis have been studied in detail. Research carried out on model systems such as *Arabidopsis, Brassica, Capsella, Triticum* and *Torenia* has expanded our understanding of the molecular regulation of the pollen tube journey, its guidance and navigation in the pistil. Recently, the critical role of the central cell in fertilization and prevention of polytubey has also been thoroughly investigated. Interesting aspects that lead to degeneration of synergids, and the factors governing degeneration, including molecular aspects have brought a paradigm shift in understanding of these intriguing units. Sophisticated confocal microscopy, live cell imaging, and molecular tools have helped in furthering our knowledge in the functioning of synergids. Recent research using the high throughput techniques have deciphered the role of various genes that regulate and govern the release of chemotropic substances, cell to cell interaction and synergid cell degeneration. Also, with the diversity displayed in form and function of organs in the angiosperms, and the switching over of roles of the cells of egg apparatus new insights have been provided on the involvement of synergids both pre- and post-fertilization. The present review provides a comprehensive account of synergids, their role in fertilization and the post fertilization events that have emerged using interdisciplinary approaches in recent years. We also discuss the variations observed in degeneration of synergids and the mechanisms that have been unraveled lately. Looking at the dynamism synergids display, newer roles of these are emerging in fertilization. It is still unknown how synergids in angiosperm taxa where genetic transformation/alteration is carried out, will respond to pollen stimuli. Since the environmental factors such as light, and temperature have a significant impact on synergids and fertilization it would be rewarding to study the role of chemo-attractants and other factors in elucidating the functional roles of synergids. Further research in developing adequate protocols for manipulating synergid functions is much desired. This research has enormous potential in the advancement of basic science and can find applications in agriculture, horticulture, and bioprospecting.

Key words: Degenerating Synergid (DSY), Filiform apparatus (FA), Persistent Synergid (PSY), Programmed Cell Death (PCD)

Introduction

Synergids, important cells of the Female Gametophyte (FG), are the primary interface between the male and female gametes (Sprunck, 2010). Earlier embryologists indicated that synergids have no significant role in fertilization but only provide guidance to the pollen tube (Maheshwari and Singh, 1967). Evidence regarding the role of synergids in fertilization was provided only after some exhaustive studies carried out on several taxa by Russell (1982) Kapil and Bhatnagar (1981) and Adhikari et al. (2020). The advent of electron microscopy provided interesting insights into the cellular and subcellular organization of the synergids (Jensen, 1965). These haploid cells arise as immediate sisters to the egg cell but exhibit reverse polarity vis-à-vis egg cell (Sprunck, & Groß-Hardt, 2011). Initial development of both the synergids follow the same pattern but towards the maturity these show differences in structure and functions. One of the synergids (that degenerates first) (DSY) undergoes programmed cell death (PCD) as it prepares to receive the pollen tube. The trajectory of the (DSY), also known as receptive synergid, has been followed by scientists and is well understood (Jensen et al,. 1977, Johri and Ambiogokar, 1984). DSY attracts and guides pollen tube entry into the embryo sac/FG besides facilitating pollen tube effusion. The other synergid, referred to as persistent synergid (PSY), has a short life span post fertilization, remains inflated and intact at the time of pollen tube entry (Coimbra and Salema, 1999). Completion of double fertilization triggers rapid elimination of the persistent synergid, an important event in checking polytubey. Interestingly, *Arabidopsis* can reverse polytubey block when the egg cell or central cell remain unfertilized, allowing the second pollen tube to recover the early fertilization failure (Beale et al., 2012; Kasahara et al., 2012; Maruyama et al., 2015). The synergids in apomictics and non-apomictics are also being examined to further elucidate their role in syngamy as well as double fertilization (Musiał & Kościńska-Pająk, 2013). Synergids show some anomalies and subcellular structures that are not characteristic of amphimictics (Lawit et al., 2004). It is now well understood that the signaling molecules and specific phytohormones have a central role in striking a molecular dialogue between male and female gametophyte (Boisson-Dernier,

2011). Many ions such as calcium are known to mediate response (Higashiyama, et al., 2003). More information has been added to the role of transcription factors, protein biosynthesis and metabolic pathways (Yue et al., 2014). The role of metabolome in governing the pollen tube pathway and delivery is equally important to understand the underlined mechanisms that govern the pollen tube entry (Nägele et al., 2017).

The last few years have focused on the identification and elucidation of the role of various genes that govern fertilization via synergid mediated cascading events making synergids an attractive system for genetic studies Li et al. (2016). The molecular tools have indicated a complex networking of genes between male and female gametophytes. The present review highlights the recent insights at the cellular, ultrastructural, and molecular level in these important units of the female gametophyte. Here we provide a comprehensive account of processes operating at the molecular and transcriptome level. However, there are many aspects that need intricate experimentation. Despite, the efforts made by many researchers, it is still unknown how synergids in angiosperm taxa, where genetic transformation/alteration is carried out, will respond to pollen stimuli and other phenoevents. Besides, many aspects of change in the cell fate vis-à-vis synergids in the female gametophyte need further investigation.

Synergids: structure, form, and functions

The synergids, generally two in number, are formed soon after megagametogenesis. The shape and size of synergids vary in different plants (Wilms, 1981). Dimorphic synergids are reported in *Allium tuberosum* (Deng et al 2016) where the smaller is the receptive or degenerative synergid and the larger remains persistent. The number of synergids may be one as in *Peperomia* type; or may be absent as (as in *Plumbago* and *Plumbagella*). This variation is attributed to the early steps of megasporogenesis and divisions during megagametogenesis (Maheshwari amd Negi, 1955). The synergids are characterized by the presence of wall ingrowths or the filiform apparatus (FA**)** which is a structurally and physiologically important part at the micropylar end. The site of attachment of the FA in synergids runs along the length of the common wall as seen in *Nicotiana* (Mogensen and Suthar, 1979), *Petunia* (Van Went, 1970), *Proboscidea* (Mogensen, 1978), and *Helianthus* (Newcomb, 1973; Yan et al.,1991). FA shows morphological diversity; in *Stipa* the convolutions are spread across and the synergid cytoplasm; massive proliferation of wall material into the synergid has been observed in *Brassica* (Sumner and Van Caeseele, 1989), *Capsella* (Schulz and Jensen, 1968), *Gossypium* (Jensen, 1965), *Populus* (Russell et al., 1990), and *Spinacia* (Wilms, 1981). In *Jusione, Torenia* and *Saintpaulia* filiform apparatus is exerted from the integuments and exposed directly to the interior of the ovary. The typical FA may be absent from the synergids of some members of the family Asteraceae, including *Calendula officinalis*, *Cichorium intybus, Crepis tectorum, Picris echioides* (Godineau, 1969), and *Crepis capillaris* (Kuroiwa, 1989). However, the thickenings

of the synergid wall in these genera may still carry out the functions of the FA. Histochemical localization has pointed at the presence of polysaccharides in *Capsella* (Schultz and Jensen, 1986) and hemicellulose in FA of *Paspalum* (Chao, 1971). Regarding the presence of FA in synergids of apomictic species, earlier it was hypothesized to be absent, but the work carried out by Płachno et al. (2014) revealed that same was not true for Asteraceae.

Jensen et al., in his classical paper (1977) revealed that synergids have uneven cell wall, which is thickest at the micropylar end, becoming discontinuous or almost absent at the chalazal end. This discontinuity facilitates the sperm nuclei to reach the female gametes (Willemse and Van Went (1984), Kasahara et al (2005). Russell (1993) and later Puwani and Drews (2008) while studying the synergids divided them into three subzones namely, the synergid hooks zone I, the neck-like zone II and the head-like zone III. Zone I is the chalazal end of synergid which is wrapped by central cell cytoplasmic protrusions, the synergid hooks or central cell apical pockets. Zone II, which is neck-like in shape, parallels the synergid hooks that are in the form of a complete ring around the two synergids. Zone III is the micropylar end and is most accessible to the advancing pollen tube and the part that lies external to the central cell pocket has a filiform apparatus.

Cytoplasmic organization

Synergids show reverse polarity with respect to egg cell in the same FG. The young synergid cell has numerous small vesicles or large vacuoles and dense cytoplasm (Folsom & Peterson, 1984). Gradually, the vesicles fuse to form a single large vacuole as in *Capsella* (Schultz and Jensen, 1968) or several small ones as in *Zea* (Diboll and Larson, 1966) or one large and several small vacuoles spread uniformly, as seen in *Nicotiana.*

The vacuoles with high calcium content, up to 50% of the dry weight act as a chemical signal for pollen tube attraction and entry into the embryo sac (Jensen, 1965; Chaubal and Reger, 1992a, b). Higashiyama (2002) observed that the mature synergid cytoplasm is densely occupied by endomembrane compartments (mitochondrion, ribosomes, dictyosomes, ER and plastids). This organization is reflective of a highly active secretion system generating messenger molecules towards the micropylar end where chemotrophic attractants are synthesized. The organelles in mature synergids in some Poaceae members show a polarized distribution: plastids near the chalazal end and most of the mitochondria and dictyosomes at the micropylar pole with ER and nucleus in close association and dispersed ribosomes (Jane, 1997). Vacuolation towards the chalazal pole of persistent synergid post pollination was also observed by Jane (1997). Ultrastructure of synergids in some apomictic species display changed orientation in

microtubules (Greehaam and Chapman, 1990). Persistent synergids showed increase in number of mitochondria, plastids and ribosomes and facilitated nutrient transport in *Beta vulgaris* (Li, 2014). Plachno et al. (2014) compared synergid morphology and ultrastructure of *Taraxacum tenuifolium* (normal amphimictic) with the apomictic tetraploid *Taraxacum brandenburgicum* and found synergids in both species possessed a filiform apparatus. However, in *T. brandenburgicum*, both synergids were persistent even after the formation of embryo and endosperm suggesting some anomalies occur post fertilization. Secretory structures in the vicinity of the filiform apparatus including lipid bodies and starch grains were observed in synergid cytoplasm *S. rupestre* (Brzezicka and Kozieradzka, 2021)

Synergid haustoria

Development of haustoria and other haustorial structures arising as branches and buds from reproductive cells is a common feature in angiosperms. Synergid haustoria have been reported in the Asteraceae members e.g., *Calendula, Cortedaria, Cotula*, *Mutsia,* and *Ursinia* (Davis 1962 an Philipson, 1977). Presence of extensive synergid haustorial structures have been reported in Santalaceae e.g., *Quinchamalium*. In *Cortedaria,* the micropylar end encroaches into the nucellus and contains numerous transfer cell walls (Philipson, 1981). Crassulaceae members are also known to have elongated and extensive synergid haustoria. The nutritive role of persistent synergid haustorium with elaborate cell wall ingrowths after fertilization were observed by Huang and Russell (1992, 1994). In *Cotula australis* synergid itself acquired haustoria-like structure and presence of finger- like projections in the haustoria suggesting their role in transfer of nutrients (Johri and Ambegokar, 1984b).

Functions

Synergids play a pivotal role in double fertilization, facilitating the fusion of one male gamete with the egg cell and the fusion of second male gamete with central cell (either with the polar nuclei or the fused product of polar nuclei, the secondary nucleus). The primary functions are attraction of the pollen tube towards the micropyle, its guidance to the female gametophyte and intercellular communication during pollen tube reception (Higashiyama et al., 1998). The pollen tube grows along placental surface then to the funicular surface before it enters micropyle and finally female gametophyte. According to Punwani et al. (2007) the female gametopyte guides pollen tube at placental to funiculus and guidance from funiculus to the micropyle. According to Shimizu and Okada (2000) funicular guidance signals and micropylar guidance signals help the pollen tube to grow from the funiculus to the micropyle. Funicular guidance is controlled both by sporophytic and gametophytic tissues that operates through ovular signals (Dresselhauz and Franklin-Tong, 2013) and micropylar guidance is regulated by chemical signals that ensure a short-range pollen tube attraction. Higashiyama et al (2001) demonstrated that a single

synergid cell was sufficient to generate attraction signal however, the presence of two cells compounded the effect. The presence of active dictyosomes and their cisternae in synergids indicate their role in secretion. Several studies indicated FA as the site of pollen tube entry however, Leshem et al. (2013) have demonstrated that the pollen tube does not enter directly into the synergids through the filiform apparatus. It grows through cell wall invaginations beyond FA into a zone of SC (synergid cell) where the pollen discharge occurs. Though the FG of sexually reproducing plants has been well investigated, the details of FG in plants with asexual seed formation have only started to emerge (van Baarlen et al., 2002).

Synergid apogamy has been observed in a few taxa where specialized synergid cells give rise to embryos. In *Oryza sativa* (rice AP III), the role of synergids in embryo formation has been well explained (Mu et al., 2010). Nutritional role of synergids has also been studied as these have active machinery for synthesis of plethora of nutrients that support the growth of other cells of the FG as well (Plachino and Swiatek, 2012). Even in orchids where the endosperm is absent, the role of synergids have been illustrated (Alvarez & Sagawa, 1965).

Synergids: A fresh look and insights using molecular approaches

Recent molecular studies have resulted in understanding the role of various genes responsible for morphogenesis, differentiation, functions and degeneration of synergids. A large number of genes, for secretion are expressed in synergids (Ohnishi et al., 2011). The research has revealed that cell to cell communication between the two synergids is extremely important for their proper functioning and subsequent fertilization. The studies are supported by the work on myb 98 mutant. In such mutants one of the synergids acquires egg cell fate. It is the communication between two synergids which restricts only one synergid cell to become egg cell, the other synergid continues to produce attractants for the pollen tube (Susaki et al., 2021). This cell-cell communication works fast and helps decide to which of the two synergids should acquire the egg cell fate. Genes coding extracellular signaling molecules expressed preferentially in the synergids is a characteristic of dicots (Jones-Rhoades et al., 2007).

The sequence of fertilization involves: the final entry of pollen tube into the synergid; arrest of growth of pollen tube; mutual demise of pollen tube and receptive synergid and finally, the delivery of male gametes. The precise crosstalk between male gametophyte and FG is a prodigious event involving several genes and pathways operating in a coordinated manner. The signaling system helps synergids and pollen tube to sense their mutual proximity which is a fatal attraction leading to death of the two – pollen tube and the receptive synergid. The female gametophyte communicates with the incoming pollen tube via synergids, and this interaction regulates and slows down pollen tube growth, finally arresting the growth. The cross talk between synergids (female gametophyte) and pollen tube (male gametophyte) culminates in the bursting of the pollen tube which also witnesses a simultaneous degeneration of the receptive synergid. This act of a

mutual demise is distinctive of angiosperms. Bursting of the PT (pollen tube) is a critical event in the sexual phase of the plant and must occur with great precision. The reproductive success depends on the integrity of pollen tube that is maintained through the style and its bursting at the right time and place upon its arrival in the receptive synergid. Bursting too soon or failing to burst when it should, results in a reproductive failure. A few key female factors such as LURE peptides and FERONIA life TFs controlling "pollen tube reception" which instruct the cessation and subsequent discharge of the penetrating pollen tube, leading to sperm release have been identified (Johnson and Preuss, 2002: Kessler and Grossniklaus, 2011; Drews and Yadegari 2002: Berger et al., 2008). These factors are FERONIA (FER) (Escobar-Restrepo et al., 2007, Huck et al., 2003), NORTIA (NTA) (Kessler et al., 2010), LORELEI (LRE) (Tsukamoto et al 2010), and early nodulin-like proteins (ENODLs) (Hou et al., 2016). Factors like HERCULES RECEPTOR KINASE1 (HERK1) and ANJEA (ANJ) are strongly localized at the filiform apparatus of the synergid cells and mediate pollen tube reception (Lopes et al 2019). TURAN and EVAN are also synergid expressed genes required for pollen tube reception (Linder et al., 2015). Though the role of MyB98 in FA formation is well documented, it is now known to affect morphology and cellular dynamics of the synergid cells (Kasahara et al., 2005). MyB 98 which is seen in the synergid cell nuclei is known to bind to specific sequence of the DNA. It acts as a transcriptional regulator with 16 downstream genes of which at least one DD11 is reported to be a target of My B 98 (Punwani et al., 2007). DD11 can bind to MYB98 and thus activating the expression of synergid-gene regulatory network. This activation of a network of genes is responsible for guiding the pollen tube and also for formation of the filiform apparatus. MyB98 is also required for the production of chemoattractants for pollen tube (Kashara Et al., 2005). LRE and FER interact to receive pollen tube in the female gametophyte, FER encodes receptor like kinase LRE (Lorelei) and NTA (Nortia). LRE interacts with FER in the lumen of the endoplasmic reticulum acting like a chaperone and brings FER to filiform apparatus (Li et al., 2015). In FA, LRE acts as a compressor with FER and perceives signals given by pollen tube. The changes in the calcium profile are then triggered, besides production of ROS (Ngo et al., 2014). One of the pollen tubes enters synergids, LRE inhibits further growth of the pollen tube through a signal cascade. The LRE participates in the pollen tube receptor by both initiating and reducing its growth after it interacts with the synergids. According to Rotman et al. (2008) the pause in pollen tube growth may then activate additional signaling between pollen tube and synergids which then completes pollen tube reception. The LRE has two functions to play; chaperoning FER in the ER enroute to FA and to act as a coreceptor with FER in FA (Li et al., 2015).

The pollination stimulus also brings about a ROS (reactive oxygen species) spike inside the female gametophyte (Martin et al., 2013). These reactive species from the female gametophyte bring about pollen tube rupture and are generated from NADPH oxidases (NOXs) in the female gametophyte (Duan et al., 2014). The interactions between RAC/ROPs (RHO-type GTPases; Ras homologous

proteins) and FER and LRE mediate the activation of NADPH oxidase for ROS generation (Duan et al., 2014). A signaling pathway comprising FER-RAC/ROP-NADPH oxidase-ROS between the pollen tube and female gametophyte is required and LRE is also a part of the signaling pathway making it too intricate (Li, et al., 2015; Nissen et al., 2016). Thus, FER interacts with ROPGEFs that activate RAC/ROP complex for signal response. This interaction brings about formation of GTP from GDPRALF34 in the inner integument binds to the BUPS/ANX receptor complex in the pollen tube. RALF34- BUPS/ANX receptor complex ruptures pollen tube in the synergid cells. FERONIA homologues ANXUR 1and ANXUR 2trigger the rupture of the pollen tube facilitating sperm delivery in the female gametophyte. Some male transcription factors involved in pollen tube reception are MYB97, MYB101 and MYB120. Kasahara et al. (2005) and Marton et al. (2005) identified genes MYB98 and ZmEA1 respectively in *Arabidopsis* and Maize. In *Arabidopsis* MYB98 gene is expressed predominantly in the synergids and regulates the expression of genes required for filiform apparatus and is known to encode an R2R3-MYB transcription factor. Model systems such as *Nicotiana* and *Arabidopsis* are being studied to decode other attributes by plant scientists.

A network of several molecules has been found that play an essential role in the pollen tube journey which includes pollen tube guidance and reception. The receptor molecules such as Buddha Paper Seal 1 and 2 (BUPS1/2), ANXUR 1 and 2 (ANX1/2) guide the entry of pollen tube. Several small peptides known as Rapid Alkalinization Factors (RALF) 4, 19 and 34 as their ligands - molecules modulate the receptors' functions (Ge et al., 2019). While the receptors and RALF4 and 19 are required to maintain pollen tube integrity during the growth process, RALF34, expressed in the female facilitates the bursting process (Somoza et al., 2021). However, the role of multitasking FERONIA appears central in the entire event (Li et al., 2016).

Li et al. (1996) suggested that Rop/Rac activates tip growth by acting upstream of Ca^{2+} and may regulate the tip-localized influx of Ca^{2+} and the formation of the Ca^{2+} gradient. Localized activation of Rho GTPases of plants (ROPs) and downstream activation of Ca^{2+} signals have been reported by Malho and Trewavas (1996).

In *Arabidopsis*, Takeuchi and Higashiyama (2012) reported specific receptor-like kinase 6 (PRK6) in the pollen tube tip. In synergids extracellular leucine-rich repeat domain LURE1/AtLURE1 that acts as an essential sensor was found. PRK6 interacts with pollen expressed ROPGEFs (Rho of plant guanine nucleotide exchange factors), facilitating pollen tube growth by activating the RhoGTPase ROP1. Thus, PRK6 is the main receptor in the pollen tube which senses AtLURE1 and activates ROP signaling.

Wang et al (2016) identified MDIS1-MIK (MALE DISCOVERER 1 -MDIS1 and MDIS1-INTERACTING RLK 1 - MIK1), a cell surface receptor heteromer present on plasma membrane. These are kinase containing extracellular leucine-rich repeats and an intracellular kinase domain

which perceive/sense the AtLURE 1 attractant. AtLURE1 binds to the extracellular domains of MDIS 1- MIK. Two novel members *TURAN* (*TUN*) and EVAN (EVN) are also identified in the pollen tube reception pathway. These encode a uridine diphosphate (UDP)-glycosyltransferase superfamily protein and a dolichol kinase respectively, both required for N-glycosylation in ER present in the pollen tube (Lindner et al., 2012).

Glycosylphosphatidylinositol-anchored protein LORELEI (LRE), LLG1 and early nodulin- like protein functions (ENODLs) seem to be the co-receptors for FER signaling at the entrance of female gametophyte. These regulate the activity of RBOHs and ROS generation in synergid. FERONIA interacts with ROP-guanine nucleotide exchange factors (RopGEFs, where Rop is Rho-like GTPases from plants) and brings about formation of GTP from GDP. This activates RAC/ROPs that direct the pollen tube growth. FERONIA/SIRÉNE (FER/SRN), a receptor-like serine/threonine kinase present in the filiform apparatus, is probably the first protein to be involved in pollen tube reception event (Huck et al., 2003; Escobar-Restrepo et al., 2007). Accumulation of NTA in Golgi apparatus is seen during synergid differentiation and in FA during pollen tube reception (Jones et al., 2017). It controls the synergid activity in response to extracellular ROS present in the micropylar end. LORELEI (LRE) is a glycosylphosphatidylinositol (GPI)-anchored protein, predominantly expressed in the synergid cells.

Role of the central cell in guiding the pollen tube functioning has also been understood. A protein CENTRAL CELL GUIDANCE (CCG) present in the central cell and not reported from the synergid or egg cell is involved in regulating pollen tube guidance mechanism. It co-regulates CRPs through a set of other interacting genes, namely CCG BINDING PROTEIN1 (CBG1), mediator complex (MED), and central cell-specific AGAMOUS-transcription factors including LUREs (Li, et al., 2015). According to Chen et al (2007), this protein is alone sufficient for providing pollen tube guidance, indicating the critical role of central cell in pollen tube guidance. FERONIA interacts with ROP-guanine nucleotide exchange factors (RopGEFs, where Rop is Rho-like GTPases from plants) and brings about formation of GTP from GDP. This activates RAC/ROPs which are regulators of the pollen tube.

Recently, several members of the *Cr*RLK1L family have been identified as receptors for RALF peptides. While, FER for RALF1 and RALF23, ANX1/2 and BUPS1/2 have been related to RALF4 activity, THE1 (THESEUS1) is a pH-dependent receptor for the peptide rapid alkalinization factor (RALF) 34 (Gonneau et al., 2018). This signaling module has a role in the fine-tuning pollen tube bursting, as THE1 also binds to ANX1/2 and BUPS1/2 receptor kinases, which form a complex in the pollen tube. RALF34 is expressed in the ovule and not in the pollen tube but competes with pollen-tube-specific RALF4 and RALF19 for binding to ANX1/2 and BUPS1/2 to regulate pollen tube growth and sperm cell release. RALF34 therefore may be

considered a spatial paracrine signal given from the female gametophyte. It interferes with the autocrine cell wall integrity maintenance system, triggering pollen tube rupture and release of sperm cells (Ge et al 2017).

The final step involves a signal from endosperm to synergid nucleus when the identity of the synergid cell completely disappears due to the nuclear disorganization during endosperm proliferation. This step is regulated by FIS-PRC2 (FERTILIZATION-INDEPENDENT SEED-Polycomb Repressive Complex 2), an endosperm-specific polycomb gene silencing complex specific to the central cell and the endosperm (Köhler et al., 2012). This implies that polytubey block is activated by central cell fertilization through the FIS-PRC2 pathway.

How and why of DSY demise?

Synergids or the siren cells attract the pollen tubes once they are through with their journey in the style. This interaction leads to degeneration of the receptive synergid by programmed cell death, a key step during pollen the tube reception (Russell, 1993; Higashiyama, 2002). The degenerated state sustains itself till cessation of pollen tube growth and the release of the pollen tube contents (van Went and Willemse,1984). This has an evolutionary implication in terms of increasing control of the sporophyte over the gametophyte (Lora et al 2016). In derived angiosperms, cues for pollen tube guidance toward the FG is provided by the outer integument (Herrero, 2000, 2003). Degeneration of synergids can also occur pre-pollination due to some ontogenetic changes (Li et al 2009). Synergid degeneration in Arabidopsis (Leydon et al., 2015), barley (*Hordeum vulgare*) and pearl millet (*Pennisetum glaucum*) occur in the absence of pollination (Engell, 1988; Chaubal and Reger, 1992). However, in most plants, the programmed cell death (PCD) of the PT and one synergid happen simultaneously suggesting that synergid degeneration is influenced by the pollen tube signal (Russell 1992, Drews and Yadegari, 2002). In a few plants however, synergid degeneration is triggered after coming in direct contact with the pollen tube (Russell 1992, Sandaklie- Nikolova et al. 2007). Therefore, it is still not completely resolved whether the pollen tube discharge is an absolute requirement for receptive synergid degeneration. According to Russell (1992) and Higashiyama et al. (2000) pollen tube discharge is mechanical and may occur due to massive increase in volume and pressure, resulting in a bursting of the synergid membrane in *T. fournieri* (Higashiyama et al., 2001). The synergid demise invariably involves a dramatic decrease in cell volume, collapse of the vacuoles, and complete disintegration of the plasma membrane and most cell organelles (Huang et al., 1993). However, several lines of evidence suggest that synergid degeneration does not result from mechanical breakdown of PT in *Arabidopsis*. Amien et al. (2010) reported the presence of a synergid-expressed defensin-like (DEFL) protein, ZmES4 that interacts with the KZM1 (potassium channel) present in pollen tube in maize. Subsequent interaction between

ZmES4 and KZM1 results in channel opening and K^+ influx which leads to water uptake and culminates in osmotic pollen tube burst. As ZmES4 is involved in pollen tube bursting it gets degraded soon after fertilization.

According to Kessler and Grossniklaus (2011) the pollen tube and synergid coordinate their mutual demise, and in the process male gametes are delivered for double fertilization. An elaborate machinery of the male gametophyte residing near the pollen tube tip is activated and establishes communication with the cells of FG, leading to its burst. In *Arabidopsis,* parallel to the FERONIA signaling pathway, molecular events involving AGPs play an active role in death of the receptive synergid. The AGPs are arabinogalactan proteins consisting of a large family of hydroxyproline-rich proteins, anchored to the plasma membrane and extremely rich in sugars. The expression of AP1G, the γ-subunit of the tetrameric ADAPTOR PROTEIN1 (Adaptor protein complexes are key regulators of cargo sorting into vesicles) is involved in acidification of the vacuole, an important mechanism in synergid degeneration (Wang et al., 2017). AP1G is crucial for synergid-controlled pollen tube reception and mediates synergid degeneration through V-ATPases, enzymes that mediate vacuolar acidification. According to Schumacher and Krebs (2010) the acid content in vacuoles initiates proton homeostasis, which further affects endomembrane trafficking through ROS and Ca^{2+} spiking. Direct evidence regarding role of ROS in synergid cell degeneration is still lacking (Sankaranarayanan et al 2020). As mentioned earlier FERONIA is required for maintaining high ROS at the micropylar end in the ovule. It has been seen that ROS accumulation occurs around the filiform apparatus which also has FERONIA (Duan et al., 2014). FERONIA is known to induce RBOHD- dependent ROS production via the GEFs and ROP. Respiratory burst oxidase homologue H (RBOHH) and RBOHJ are regulated by receptor-like kinases (RLKs) such as ANXUR1 and ANXUR2, colocalized in the same plasma membrane domain at the pollen tube tip. They act downstream of ANXs to control ROS production during PT growth (Boisson-Dernier et al., 2013). By regulating RBOHH and RBOHJ, pollen tube integrity is maintained.

The synergid cell death module also requires a heterodimer VAL-VDD (VALKYRIE-VERDANDI). VDD and VAL are transcription factors of the family REM (Reproductive Meristem) as reported by Mantegazza et al. (2014). These are direct targets of ovule identity complex - STK-SEP3 (SEEDSTICK-SEPALLATA3). VAL-VDD heterodimer is involved both in PCD of receptive synergid and pollen tube death by bursting (Mendes et al 2016). This controls downstream expression of mitochondrial chaperon - GFA2 (gametophytic factor 2) which is responsible for the mitochondrial protein folding as pointed by Christensen (2002). Thus, multiple factors are operating in the demise of DSY each following its own pathway with downstream cascading events.

Persistent Synergid (PSY) the Journey forward

A single pollen tube delivers two sperms for double fertilization resulting in embryo and endosperm formation. Once this is achieved, polytubey (the phenomenon of entry of multiple pollen tubes in the FG) is checked through polytubey block (Beale et al., 2012; Beale and Johnson, 2013). Upon successful fertilization, the persistent synergid cell (PSY) is destined to die. The DSY undergoes PCD when receiving pollen tube discharge, while the persistent synergid cell, undergoes nuclear degeneration within a few hours after successful double fertilization (Beale et al., 2012; Vo lz et al., 2013). Chromosomal condensation and the loss of nuclear envelope integrity has been observed after the SE (synergid-endosperm) fusion by (Maruyama et al.,2015). Several workers, Schulz and Jensen (1968), Beale et al. (2012), Völz et al. (2013) have indicated nuclear disorganization as a feature of synergid inactivation. Even though the milieu in which persistent synergid nucleus and the endosperm nuclei lie is common, only the PSY nucleus is selectively eliminated during the SE fusion. In many cell types where the programmed cell death occurs, nucleases are thought to have a role in nuclear degeneration (Ito and Fukuda, 2002; Furuta et al., 2014). However, the mechanism involved in death of the persistent synergids appear to be different as the elimination in this case is selective. Alternatively, the selective nuclear elimination may be caused by premature chromosome condensation. Studies with artificial fusion between two cells at different stages demonstrated that M phase of one cell induces premature chromosome condensation of chromosomes (Rao and Johnson, 1972; Szabados and Dudits, 1980). However, this aspect of premature chromosome condensation and how selective elimination of persistent synergid is made possible, needs to be investigated. The precise quantification of DNA content in synergid nucleus before and after fertilization might throw some light on the process. It is however certain that for degeneration of the persistent synergid cell completion of double fertilization is a pre-requisite. In Arabidopsis thaliana, fusion of the persistent synergid with the endosperm leads to cytoplasmic dilution of pre-secreted pollen attractants. This leads to nuclear degeneration, followed by rapid inactivation of persistent synergid. The entry of multiple pollen tubes is therefore discouraged into the female gametophyte. Maruyama et al. (2015) traced the sequences as: i. Pollen tube attraction is terminated by the inactivation of persistent synergids, ii. Persistent synergids are fused with the fertilized central cell or endosperm, iii. The fertilized egg regulates synergid nucleus degeneration via ethylene signaling and iv. Polycomb proteins and an AGP are required for synergid nucleus degeneration.

Calcium the Key Player

The ‘male germ unit’ in flowering plants is organized between the two sperm cells and the vegetative nucleus forming a functional association (Dumas et al., 1984). This assemblage favours the transportation of the male gametes within the tube and ascertains their simultaneous delivery

to female gametes. But this assembly (association) must be disturbed, and the two sperms are dissociated to enable their union with the egg cell and the central cell. Because the assemblage is presumably maintained by cytoskeletal elements (Palevitz and Tiezzi, 1992), calcium in the synergid may be involved in the breakdown of the cytoskeletal elements and the preparation of the sperm cell surface for fusion. The egg cell during the process retains consistently low levels of calcium (Chaubal and Reger, 1992a, b; Tian and Russell, 1997; Yu et al., 1998; Tian et al., 2000). However, in *Plumbago zeylanica* where synergids are absent, the egg has high calcium levels at maturity (Tian et al., 2000). This observation further elucidates the role of calcium in pollen tube attraction to the ovule and its entry into the FG *in vivo* (Cass and Karas, 1974; Russell, 1982). ACA9 is another Ca2+ transporter present in the pollen tube which presumably interacts with ZmES4, the evidence of which is however, awaited (Staiger et al., 2010).

Live cell imaging studies by Ngo et al. (2014) on *Arabidopsis* provide a conceptual framework for the molecular mechanism of the multistep programmed cell death. The live imaging studies have intricately revealed the role of Ca^{2+} pattern in three interacting cells – Pollen tube (PT), DSY and PSY during the phase of pollen tube discharge. These patterns have been traced down to four stages of PT growth and sensing of the mutual proximity between PT and synergids. Phase I is - slow PT growth, when pollen tube grows slowly along micropylar region of the two synergids. This initiates calcium spike in both synergids but with different intensities. Pollen tube also shows local oscillations at the tip region. Phase II is marked by fast PT growth with elevation of Ca^{2+} at the tip. In PSY oscillations continue while in DSY, the cell gets flooded with calcium. By the time pollen tube reaches chalazal pole of DSY, calcium spike is observed. At the chalazal pole of the degenerating synergid, the pollen tube stops growing for a while, but as it moves towards the micropylar pole of the DSY, the pollen tube growth is fast. Here for a short while pollen tube growth stops but is resumed as fast growth towards micropylar pole of DSY. Phase III - PCD is characterized by the rupture of the PT tip and the collapse of the DSY and marked increase in calcium in PT. The calcium is higher in PSY than DSY, but it soon subsides. The Phase IV is marked by oscillation recovery in PSY. From here onwards, the calcium signatures in PSY (ready to degenerate) follows the same pattern as of the DSY. Thus, calcium dynamics in the DSY in response to pollen tube growth distinguishes it from its genetically identical sister cell, the PSY.

Conclusions

The present review dwells upon the intricacies of the role of synergids in fertilization. It highlights the role of genes and signaling cascades that lead to the PCD of degenerating synergids and elimination of the persistent synergids. The role of calcium signaling is exemplary, as calcium plays a key role in mitigating the pollen tube growth and through the central cell impacts the

fertilization process. The small molecules and transcription factors interplay also has an impact both pre and post fertilization. LRE, LLG1 and early nodulin-like protein functions (ENODLs) are the co-receptors for FER signaling at the entrance of female gametophyte. The responses triggered in PSY, time lapse between the degeneration of the two synergids, and nuclear behavior in PSY need more studies. It is also clear that the cascading pathways and feedback loops involving many drivers lead to synergid demise that essentially ensure fertilization. Diversity in angiosperm taxa warrants extensive studies in pre- and post- fertilization behavior of synergids.

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