Mitochondrion 19 (2014) 350–356

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Role of mitochondria during female gametophyte development and fertilization in *A. thaliana*

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ARTICLE INFO

Available online 7 February 2014

Keywords:
Plant mitochondria
Female gametophyte
Signaling
Reactive Oxygen Species
Arabidopsis

ABSTRACT

Plants alternate between two generations during their life cycle: the diploid sporophyte and the haploid male and female gametophytes, in which gametes are generated. In higher plants, the female gametophyte or embryo sac is a highly polarized seven-celled structure that develops within the sporophytic tissues of the ovule. It has been proposed that mitochondria are crucial in many cell signaling pathways controlling mitosis, cell specification, cell death and fertilization within the embryo sac. Here, we summarize recent findings that highlight the importance of this organelle during female gametophyte development and fertilization in plants.

1. Introduction

In plants, gametes are formed in specialized haploid structures, called gametophytes. The female gametophyte or embryo sac of flowering plants develops inside the ovule primordium where a single cell, the megaspore mother cell, undergoes meiosis (Fig. 1). Only one of the resulting haploid cells differentiates into a functional megaspore, which subsequently undergoes three mitotic divisions giving rise to an eight nuclei syncytium. Upon cellularization, the embryo sac contains two gametes: the egg cell at the mycropilar pole, and the central cell, derived from the fusion of two polar nuclei. It is also composed of five accessory cells: two synergid cells surrounding the egg cell and three antipodal cells at the chalazal pole (Fig. 1). The male gametophyte or pollen grain comprises three cells, one vegetative cell, which forms the pollen tube, and two gametic cells or sperm cells that are enclosed by the vegetative cell (McCormick, 1993, 2004). Each one of the female gametic cells is fertilized by one of the two sperm cells delivered by the pollen tube at the micropylar end of the embryo sac. The fertilized egg cell subsequently develops into a diploid embryo, whereas the fertilized central cell generates the triploid endosperm, which nurtures the developing embryo (Fig. 1). Functional mitochondria are important for each of the previously described developmental steps. Their role seems not only related with their functions in energy production. Mitochondria have been proposed as organelles intimately involved in cell signaling pathways controlling cell specification, cell death and fertilization. Despite the large number of proteins known to play important roles in female gametophyte development, we will focus this review specifically on the role of mitochondrial proteins.

2. Mitochondria distribution

Although mitochondria can be detected along the whole wild-type female gametophyte, the distribution of mitochondria inside the embryo sac seems to be not uniform, concentrating a large number of mitochondria in the central zone of the developing gametophyte and, after cellularization, in the central cell (Kägi et al., 2010; Martin et al., 2013). The egg apparatus (egg cell and synergid cells) does not contain high concentrations of mitochondria in wild-type embryo sacs.

In contrast, the gametophytic mutant *oiwa*, defective in the mitochondrial Mn-superoxide dismutase (*MSD1* gene) which shows arrested embryo sac development and abnormal egg apparatus, also shows micropylar cells with a high number of mitochondria (Martin et al., 2013). When *oiwa* embryo sacs were analyzed using a specific mitochondrial fluorescent probe (Mitotracker Red), mitochondria were not only observed around the central cell nucleus, but they were also detected at a high density in the egg apparatus cells (Fig. 2). These cells are able as well to express central cell specific markers. This correlation between central cell markers expression and high density of mitochondria strongly suggests that this last one might be a central cell feature.

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http://dx.doi.org/10.1016/j.mito.2014.01.005

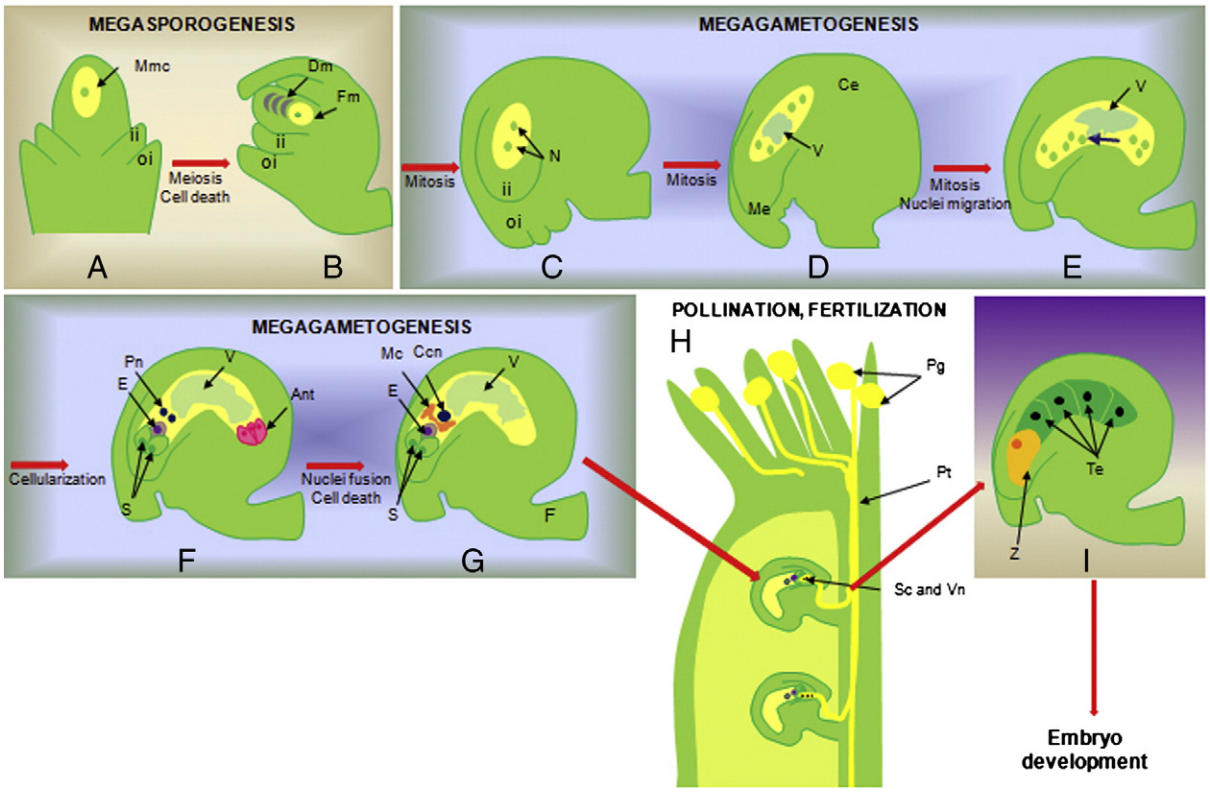

Fig. 1. Scheme showing megasporogenesis, megagametogenesis and fertilization in *Arabidopsis thaliana*. (A) Ovule primordium showing a megaspore mother cell (Mmc, yellow). The Mmc undergoes meiosis rending four megaspores. (B) Stage FG1. Three megaspores undergo programmed cell death. The scheme shows the functional megaspore (Fm, yellow) and degenerating megaspores (Dm, gray). (C) Megagametogenesis starts with three consecutive mitotic divisions. The first division results in an embryo sac at stage FG2 presenting two nuclei (N, green). These nuclei are then separated by a vacuole (stage FG3). (D) Stage FG4. Four nuclei resulting from the second mitotic division are arranged in pairs. One pair is located at the micropylar end of the embryo sac (Me) while the other pair of nuclei is located at the chalazal end (Ce) of the developing embryo sac. (E) Stage FG5. The embryo sac contains eight nuclei after the third mitotic division. One of the chalazal nuclei migrates towards the center of the gametophyte (blue arrow). (F) Stage FG6. Following cellularization and cell specification the embryo sac is now formed by seven cells and eight nuclei: Two synergid cells (green), one egg cell (purple), one central cell (yellow) containing the two polar nuclei (blue) and a big vacuole (light blue), and three antipodal cells at the chalazal end of the gametophyte (pink). (G) Stage FG7. Mature embryo sac. The two polar nuclei fused forming the diploid nucleus of the central cell (blue) and the antipodal cells undergo cell death leading to the formation of the mature female gametophyte consisting in four cells: two synergid cells (green), one egg cell (purple) and one central cell (yellow). Mitochondrial clouds (Mc) are represented in Red. (H) Following pollination, pollen grains (Pg) germinate in the stigma and pollen tubes (Pt) grow through the style and transmitting tissue towards the ovules. Each one of the female gametic cells (egg cell and central cell) is fertilized by one of the two sperm cells delivered by the pollen tube. (I) The fertilized egg cell subsequently develops into a zygote (Z) and then into a diploid embryo, whereas the fertilized central cell generates the triploid endosperm (Te). ii, inner integuments; oi, outer integuments; Mmc, Megaspore mother cell; Dm, Degenerating megaspore; Fm, Functional megaspore; Me, Micropylar end; Ce, Chalazal end; Ant, Antipodal cells; Pn, Polar nuclei; Ccn, Central cell nucleus; Ec, Egg cell; S, Synergid cells; V, Vacuole; Mc, mitochondrial clouds; F, Funiculus; Pg, Pollen grain; Pt, Pollen tube; Sc, Sperm cells; Vn, Vegetative nucleus; Z, Zygote and Te, Triploid endosperm.

The developmental role of this aggregation of mitochondria in one of the plant gametes is at least intriguing. We hypothesize that these mitochondrial clouds observed in the cytoplasm of the central cell are a feature reminiscent of the mitochondrial clouds typically seen in the animal pole of oocytes of vertebrates and invertebrates, named Balbiani's body (Pepling et al., 2007). The Balbiani body comprises mitochondria and endoplasmic reticulum organized around Golgi elements that may enable germplasm mRNAs to be specifically positioned (for a review on Balbiani body structure, formation and significance in various animal species see: Kloc et al., 2004). It would be interesting to study if central cell mitochondria are arranged in an analogous pattern and if they play similar roles in this particular plant gametic cell that is crucial for embryo development.

3. Mitochondria and ROS homeostasis in the female gametophyte

During gametogenesis, mitochondria seem to be the main source of Reactive Oxygen Species (ROS), either superoxide as well as peroxide. By using specific fluorescent probes, it was shown that mitochondrial superoxides are detected mainly in the central cell, which is consistent with the idea that the number of mitochondria is higher in this particular cell (Martin et al., 2013). Peroxides are also detected exclusively in the central cell of the mature female gametophyte, both inside and outside mitochondria. Notably, ROS are not observed around the antipodal cells, which have been showed to undergo cell death in mature embryo sacs. Since lifespan of antipodal cells seems to depend on central cell mitochondria (Kägi et al., 2010; Wu et al., 2012), the high production of ROS observed in this cell could be most likely part of a non-cell autonomous signal regulating antipodal cell death (Fig. 3).

The expression of *MSD1* gene is high during female gametophyte development and observed all along the embryo sac. However, at the time of cellularization, *MSD1* expression is confined to the egg apparatus. This pattern is consistent with the high mitochondrial superoxide and cytoplasmic peroxide production observed in the central cell. This situation excludes the egg cell to be exposed to a high oxidative status that was reported to impair further embryo development (Martin et al., 2013).

It was recently proposed that *de novo* DNA methylation in the embryo is dependent on a large population of 24-nucleotide small RNAs (sRNA) derived from the transcription of transposons through the activation of the DNA glycosylase DEMETER and the inactivation of the DNA methyltransferase MET1 (Wöhrmann et al., 2012). As transcription of retrotransposons was shown to be enhanced by mitochondrial ROS in *Saccharomyces cerevisiae* and mammals (Hitchler and Domann, 2009; Stoycheva et al., 2010), it was suggested that the oxidative environment of the central cell could trigger

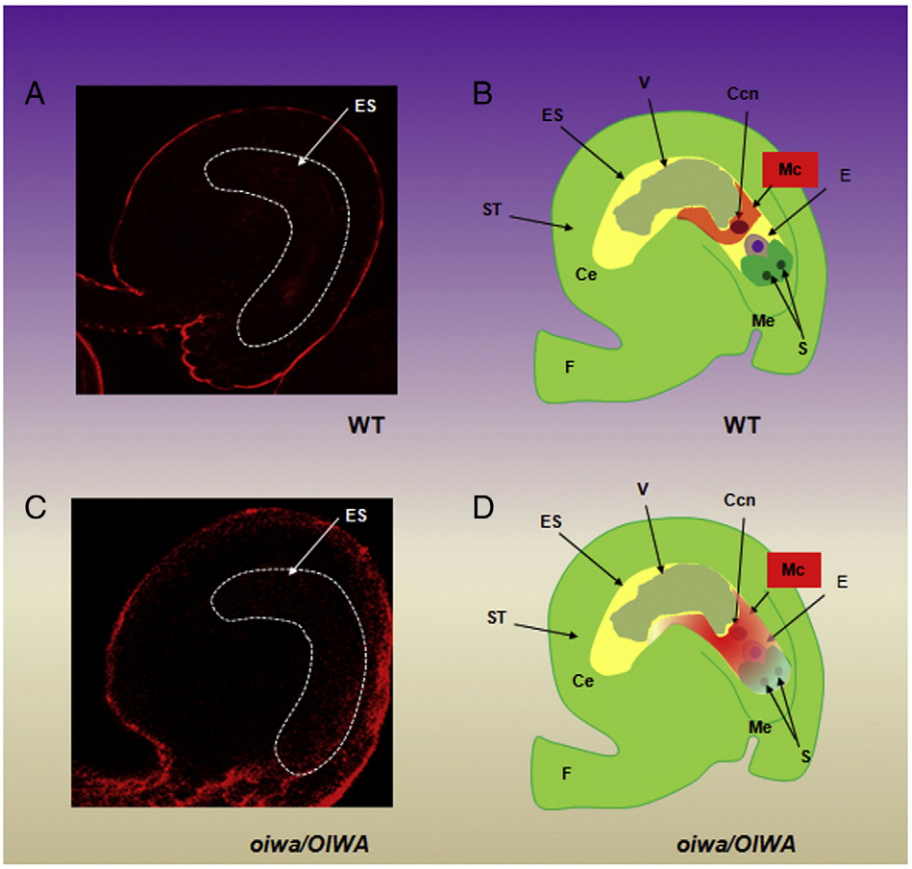

Fig. 2. Mitochondrial distribution in embryo sacs of WT (A–B) and in *oiwa* mutant (C–D) of *Arabidopsis thaliana*. A-C) Mitotracker red staining shows mitochondria distribution along the embryo sac, delimited with white dots, of WT ovule(A) and *oiwa/OIWA* ovule(C). B-D) Schematic representation showing mitochondria distribution along the embryo sac of WT ovule (B) and *oiwa/OIWA* ovule (D). ST, Sporophytic tissue (green); ES, Embryo sac (yellow); Me Micropylar end; Ce, Chalazal end; Ccn, Central cell nucleus (blue); E, Egg cell (violet); S, Synergid cells (dark green); V, Vacuole (gray); Mc, Mitochondrial clouds (orange); F, Funiculus.

transposon transcription and consequently, an increase of the sRNA population (Martin et al., 2013). Recently, a mutant defective in the *GAMETOPHYTE DEFECTIVE 1 (gaf1)* gene, encoding a putative mitochondrial protein subunit of the RNase mitochondrial RNA processing complex AtRPP30, which is responsible for tRNA processing and transcription of small non-coding RNAs shows altered gametophytic cell division (Wang et al., 2012).

Thus, the oxidative status of the central cell might be essential for influencing small RNA synthesis in the female gametophyte and *de novo* DNA methylation in the future sporophytic generation.

4. Mitosis, cell fate and mitochondrial function

Most of the gametophytic mutants affecting mitochondrial proteins show embryo sacs arrested at different stages of female gametophyte development, from the very early FG1 stage to FG5 stage, suggesting that mitochondrial metabolism or by-products are important for mitosis progression.

For example, mutants lacking ANK6, a mitochondrial protein containing ankyrin repeat motifs, show defects in female gametophyte development at the one-nucleate stage (FG1) and are eventually degraded. Occasionally, the presumed *ank6* ovules were arrested at the two-nucleate stage (FG2). In addition, ANK6 interacts with the transcription initiation factor SIG5, from mitochondria/plastids and a proportion of *sig5* embryo sacs are also arrested at early stages. The ankyrin repeat domain is likely to mediate protein–protein interactions, suggesting that more mitochondrial proteins might be required for gametophytic mitotic progression (Yu et al., 2010).

Other gametophytic mutants affecting mitochondrial proteins that present arrested embryo sacs slightly later during development, at FG3-FG5 stages, also show mitotic defects (León et al., 2007; Martin et al., 2013; Wang et al., 2012). It was proposed that ROS oscillations might regulate cell cycle progression and microtubule organization (Livanos et al., 2012a, 2012b). Ca^{+2} gradients that participate in the regulation of proteins such as cyclin-dependent kinases, mitogen-activated proteins and aurora kinases are reported to be sensible to ROS fluctuations. Concordantly, the defects observed in this class of mutants might be due to an increase of the intracellular ROS produced as a consequence of mitochondria dysfunction. This increase in intracellular ROS might affect key components of the cell division machinery. Accordingly, the formation of atypical tubulin paracrystals has been reported to occur under elevated ROS levels via mitogen-activated protein kinase activation and MAP65 phosphorylation (a microtubule associated protein) and thus directly affecting mitosis (Livanos et al., 2012b).

Many gametophytic mutants show alteration in cell specification within the embryo sac (reviewed in Chevalier et al., 2011). However, until now, only one corresponds to a mitochondrial protein defect; the *oiwa* mutant (Martin et al., 2013). As mentioned above, a proportion of egg and synergid cells acquires central cell identity in the *oiwa* mutant. All these atypical cells contain high number of mitochondria and a high oxidative status suggesting that these characteristics might be important for cell fate specification within the embryo sac (Martin et al., 2013). Abnormal egg apparatus specification was also observed in this mutant. This defect was attributed to an unusual migration of nuclei during development that might result in a different read out of the established morphogenic auxin gradient, leading to altered cell identity

(Martin et al., 2013; Pagnussat et al., 2009). The abnormal nuclei migration pattern observed in some *oiwa* embryo sacs may also account for the elevated concentration of ROS in mutant gametophytes, as ROS might also disturb microtubule organization or dynamics.

5. Mitochondria and cell–cell communication

The mechanism controlling the coordinated development of distinct cells within the embryo sac is thought to involve cell–cell communication. This type of cellular communication is mediated by physical interactions between neighboring cells and signaling molecules acting in a non-cell autonomous manner. Early studies have shown the presence of multiple plasmodesmata linking the functional megaspore and the surrounding nucellar cells, which apparently are important for the differentiation of female gametophytes (Bajon et al., 1999). An emerging picture suggests that mitochondria play a pivotal role in this communication.

In *fiona* embryo sacs, which are defective in the mitochondrial cysteinyl t-RNA synthase (SYCO), mitochondria of the central cell lacked regular cristae indicating that SYCO is necessary for the integrity of central cell mitochondria. In addition, *SYCO* expression was detected throughout the plant but exclusively in the central cell of wild-type embryo sacs by both in situ hybridization and promoter GUS reporter assays (Kägi et al., 2010). In *fiona* embryo sacs, the timing of antipodal cells' death is disrupted, which strongly suggests that lifespan of antipodal cells requires healthy central cell mitochondria (Kägi et al., 2010).

In embryo sacs defective in GCD1 (GAMETE CELL DEFECTED1), a conserved mitochondrial protein, immature gametes are observed. *gcd1* embryo sacs show a high percentage of unfused polar nuclei. Also, egg cells are consistently smaller than wild type suggesting that GCD1 is required for final development of both gamete types (Wu et al., 2012, 2013). Furthermore, as observed in *fiona* mutants, *gcd1* embryo sacs also present persistent antipodal cells. The authors found that final maturation of the egg and central cells is not required for double fertilization but is necessary for embryogenesis initiation and endosperm development. Moreover, using an elegant experiment in which *GCD1* was expressed either in the egg cell or in the central cell by using cell-specific promoters, Wu et al. (2012) were able to rescue the phenotype and function of the otherwise affected neighboring cell. These results suggest that mitochondrial function influences the reciprocal signaling between central cell and egg cell required to regulate the maturation of both types of cells (Fig. 3).

Both *fiona* and *gcd1* embryo sacs show abnormal central cell mitochondria with reduced cristae, suggesting low mitochondrial activity. Furthermore, a specific mutant in *AAC2* (a mitochondrial ATP/ADP translocator conserved between yeast and plants) also causes unfused polar nuclei, persistent antipodal cells and reduced egg cell size (Kägi et al., 2010; Wu et al., 2012). Dysfunctional mitochondria were also observed in *oiwa* gametophytes by using a specific fluorescent probe (JC-1, Hauser et al., 2006) which estimates the mitochondrial membrane potential (Martin et al., 2013).

A significant number of studies have demonstrated the importance of mitochondria in karyogamy. This process occurs three times during the angiosperm life cycle. The first karyogamy event takes place when

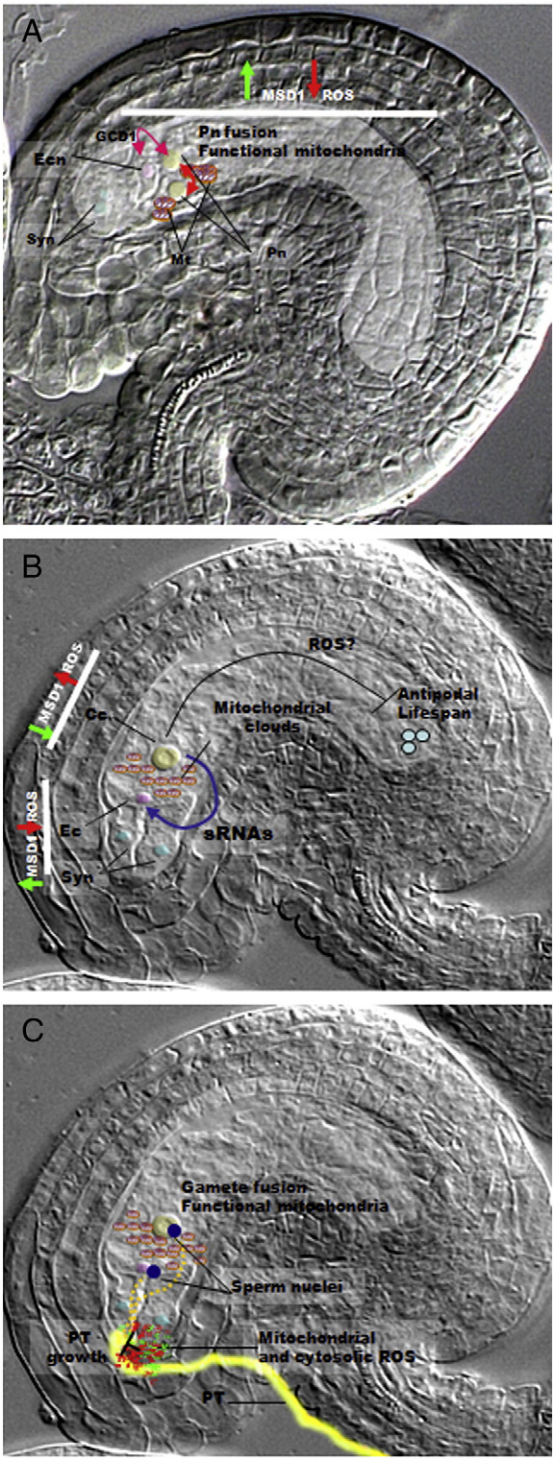

Fig. 3. Proposed mitochondrial signals during female gametophyte development and fertilization. (A) DIC picture of an ovule with en embryo sac at stage FG6 showing mitochondrial signals suggested to be involved in polar nuclei fusion and egg cell and central cell maturation. Functional mitochondria are required for both events. GCD1 was shown to be required for the reciprocal signaling between central and egg cell essential to regulate the maturation of both types of cells. Mitochondrial ROS cannot be detected at this stage by conventional techniques while mitochondrial MSD-1 is upregulated along the whole embryo sac. (B) DIC picture presenting an embryo sac at FG6–FG7 stage showing mitochondrial signals proposed to be involved in embryo sac maturation. Antipodal cell death might be controlled by a noncell autonomous mechanism for which functional mitochondria are required. ROS were suggested to be the putative signal coming from the central cell that regulates antipodal lifespan. At this stage, both mitochondrial superoxide and cytosolic ROS are detected exclusively around the central cell nucleus. *MSD1* expression was shown to be upregulated in the egg apparatus and downregulated in the central cell. The position of the observed mitochondrial clouds is shown around the central cell nucleus. Antipodal cells are showed as circles in the chalazal pole of the embryo sac. The blue arrow indicates the sRNA population that is proposed to migrate from the Central cell to the Egg cell. (C) DIC picture showing a mature embryo sac in which the mitochondrial events suggested to be triggered by pollen tube arrival are schematized. Mitochondrial and cytosolic superoxides are detected inside synergid cells at the time of pollen tube arrival. This oxidative burst might be involved in pollen tube growth inhibition and rupture, essential for sperm cells release. Central cell is fertilized by one sperm cell to give rise to the triploid endosperm, and the egg cell is fertilized by the second sperm cell to form the diploid zygote. Following plasmogamy, karyogamy is also thought to depend on mitochondrial signals (ANK6 and SIG5 pathway). Red and Green dots indicate mitochondrial and cytoplasmic ROS, respectively. Cc, Central cell; Ec, Egg cell; Ecn, Egg cell nucleus; Pn, Polar nuclei; Syn, Synergid cells; PT, Pollen tube; Mt, Mitochondria.

gametophytic polar nuclei fuse. The last two of these karyogamy events occur during double fertilization (Maheshwari and Johri, 1950; Weterings and Russell, 2004).

Interestingly, most of the gametophytic mutants affecting genes encoding for mitochondrial proteins show unfused polar nuclei, including *NUCLEAR FUSION DEFECTIVE1* (*NFD1*, predicted to encode the mitochondrial 50S ribosomal subunit L21), *NFD3* (predicted to encode the mitochondrial 30S ribosomal subunit S11), *NFD4* (predicted to encode a nodulin like protein) and *NFD5*, *NFD6* (encode unknown mitochondrial proteins) (Portereiko et al., 2006), *GFA2* (encodes Gametophytic Factor 2, similar to yeast mitochondrial Mdj1p chaperone) (Christensen et al., 2002), *SDH-1-1* encoding the flavoprotein subunit of mitochondrial complex II of the respiratory chain (León et al., 2007), *FIONA*/*SYCO1* (Kägi et al., 2010), *GCD1* (Wu et al., 2012) and *OIWA*/*MSD1* (Martin et al., 2013). Because these mutants show affected antipodal degeneration as well, it was proposed that defects in polar nuclei karyogamy affect the lifespan of the neighboring antipodal cells. It should be noted that this defect in polar nuclei fusion does not affect karyogamy events during fertilization, since *fiona* and *gcd1* mutants are able to be fertilized. In *fiona* mutants, one of the polar nuclei could eventually fused to one sperm cell giving rise to a diploid endosperm (Kägi et al., 2010) while in *gcd1* mutants, double fertilization is possible although early embryogenesis is disrupted (Wu et al., 2012). A similar case was observed for *oiwa* embryo sacs where a high proportion of the mutant embryo sacs was able to get fertilized, although early seed abortion was consistently detected (Martin et al., 2013). These observations strongly suggest that a complete maturation of female gametes is required for normal embryogenesis to proceed and that healthy mitochondria are essential for such maturation, contributing to a so-called "gametic factor" necessary for post-fertilization development (Wu et al., 2012, 2013).

6. Mitochondria and ROS during fertilization

Mitochondria integrity and redox control were demonstrated to be important for two additional key processes essential for sexual reproduction of plants; pollen attraction and karyogamy during fertilization.

Once pollen germinates in the stigma, pollen tubes grow through many different maternal tissues to finally reach the fully developed embryo sacs (Palanivelu and Tsukamoto, 2012). It was recently reported that pollination triggers an oxidative burst inside mature unfertilized female gametophytes. This oxidative environment inside the embryo sac is restricted to the synergid cells and is maintained until pollen tube arrival, when both cytoplasmic and mitochondrial ROS are observed (Martin et al., 2013, Fig. 3).

During fertilization, a pollen tube enters into one of the two synergid cells. This synergid cell undergoes cell death upon pollen tube entry. The pollen tube releases its contents into the degenerating synergid cell. Then, the sperm cells migrate to and fuse with the egg cell and central cell in a process called plasmogamy. Following plasmogamy, the sperm nuclei fuse with the egg cell and the central cell nuclei to produce the diploid zygote and triploid endosperm nuclei, respectively (Igawa et al., 2013).

Communication between the pollen tube and the synergid cells may initially occur via the activation of a receptor kinase complex at the surface of the synergid cells which may act as a reproductive barrier at the pollen tube reception step. Mutants such as *feronia*/*sirène* (*fer*/*srn* — lacking a receptor kinase) and *lorelei* (*lre* — lacking a putative glycosylphosphatidylinositol-GPI-anchored protein) show more than one pollen tube attracted by the mutant embryo sacs and invading pollen tubes (Capron et al., 2008; Escobar-Restrepo et al., 2007; Tsukamoto et al., 2010). These observations demonstrate the involvement of a serine/ threonine kinase receptor in the arrival/recognition of a pollen tube and in the activation of a signaling cascade (possibly through LRE and plant Rho small GTPase RAC/ROPs) that in turn activates the degeneration of the receptive synergid, as well as the release of factors affecting pollen tube growth and integrity (Duan et al., 2010). In *gfa2* mutants, lacking a putative mitochondrial chaperone, synergid cells are not degenerating, suggesting a role for mitochondria in synergid cell death and as potential targets of the FER/LRE signaling. However, because there is no fertilization in the *gfa2* mutant, an alternative model in which mitochondria are involved in a signaling pathway leading to the release of pollen bursting factors could be considered (Chevalier et al., 2011). The discovery of the mutant *abstinence by mutual consent (amc)* which displays a similar pollen tube overgrowth phenotype, but only when a mutant pollen tube interacts with a mutant female gametophyte, led researchers to think that the male–female communication required for pollen tube reception relies on some components of identical nature in both gametophytes (Boisson-Dernier et al., 2008). More recently, two pollen-expressed homologues genes, closely related to FER, were identified (ANX1 and ANX2). These proteins display a polarized localization at the plasma membrane of the pollen tube tip, and function redundantly to maintain pollen tube integrity during its growth and seem to be required to regulate the timing of pollen tube discharge (Boisson-Dernier et al., 2009, 2013).

Using live imaging of sperm cell mitochondria during double fertilization Matsushima et al. (2008) revealed that sperm mitochondria enter into the egg and central cells. These results confirmed previous observations using electron microscopy (Yamamoto et al., 2003). These observations imply that mitochondria present in both the male and the female gametophytes might have a role during fertilization. As sperm mitochondrial fluorescent signals became undetectable in the Egg (or Central) cells within 5 h after fertilization, it was suggested that they could be specifically degraded as occurs in other species (Matsushima et al., 2008).

In a mutant plant defective in the δ subunit of the ATP synthase complex, most embryo sacs develop slower in comparison to wild-type female gametophytes. In addition, this mutant also shows male gametophytic defects and embryo lethality (Geisler et al., 2012). Moreover, growth under long-day conditions is impaired in plants showing low levels of delta transcripts. These plants maintain their cellular ATP/ADP ratio and a high capacity of AOX, which indicates that considerable changes in mitochondrial metabolism are taking place. Defects in female gametophytic development in this mutant as well as in *sdh1* mutant are suggested to be due to either an uneven distribution of functional mitochondria from the megaspore during mitosis or due to varying levels of nutrients coming from the tissue surrounding the ovule (Geisler et al., 2012; León et al., 2007).

Besides their roles in embryo sac development, mitochondrial proteins ANK6 and SIG5 are important for sperm-female gamete recognition. ANK6 is highly expressed in the male and female gametophytes before and during fertilization but not after it. In *ank6* mutants, sperm cells fail to fuse with their targets (egg and central cell). On the other hand, *sig5* mutant embryo sacs that are able to reach the FG7 stage of development are not able to get fertilized (Yu et al., 2010). Additionally, *nfd1* mutants, which show unfused polar nuclei, present normal pollen tube guidance, synergid cell death, pollen tube entry, release of pollen tube contents, sperm cell migration, and plasmogamy. However, *nfd1* mutants are unable to complete the karyogamy events needed to accomplish double fertilization (Portereiko et al., 2006).

Taken together, all these results suggest that even when polar nuclei fusion and gametic karyogamy during fertilization seem to be independently regulated, functional mitochondria are required for the accomplishment of both processes (Fig. 3).

After fertilization, ROS are eliminated from the embryo sac. Female gametophytic mutants that are not able to exclude ROS from the embryo sac after fertilization show arrested embryogenesis, indicating that the regulation of mitochondrial ROS levels from the maternal side is crucial for embryo development (Martin et al., 2013).

7. Conclusions and future perspectives

Despite the large body of knowledge on female gametophyte development and sexual reproduction in plants, our understanding of the

involvement of mitochondria in cell signaling is in its early stages. Using genetic approaches, experiments have shown that specific mitochondrial functions are essential for polar nuclei fusion, nuclei migration, mitosis progression, antipodal cell lifespan, maturation of gametes, gametic nuclei karyogamy, and discrete oxidative bursts that in turn lead to correct cell specification, pollen tube attraction and fertilization (Fig. 3). Novel strategies might be undertaken to further unravel mitochondrial functions affecting embryo sac maturation and fertilization.

Detailed ultrastructure studies combined with molecular techniques to precise the location of transcripts within the embryo sac would help to understand the actual role of the intriguing mitochondrial clouds around the central cell nucleus. Do they have a similar role as the mitochondrial clouds existing within the animal egg cells? Are they important for embryogenesis or for endosperm development? Is there any deposition of mRNAs in specific zones of the cell as occur in animals? Is this accumulation of mitochondria important for *de novo* DNA methylation in the embryo?

Another intriguing point is the fact that pollination in the stigma triggers an oxidative burst inside receptive embryo sacs, most likely through a long distance signal. Although mitochondrial superoxide was not observed at early stages of this process, mitochondrial ROS are observed in synergid cells at the time of pollen tube arrival. It was suggested that an oxidative environment might be needed for pollen tube growth inhibition or rupture (Martin et al., 2013). However, what is the nature of the signal that synergid cells perceive? How is this information transmitted to mitochondria? Another important point is that this oxidative burst should be tightly controlled to allow normal embryo development. A role for mitochondrial antioxidant enzymes has also been proposed to regulate this, although more detailed work is required to better understand this process (Martin et al., 2013).

Finally, the nature of some predicted non-cell autonomous signals should be investigated, such as those triggering antipodal cell death, final maturation of gametes (egg and central cells) and nuclei recognition for karyogamy. The oxidative environment or mitochondrial metabolic status of the central cell is most likely essential for signals required for cell–cell communication within the embryo sac as well as for regulating cell identity and for controlling the lifespan of antipodal cells.

Acknowledgments

We are grateful to the International Conference of Plant Mitochondrial Biology-2013 for kindly inviting this review. A.B., J.P.C and D.S are doctoral fellows of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). M.V.M. and A.M.D are postdoctoral fellows of CONICET. E.Z. and G.C.P. are CONICET researchers. The research was funded by the Agencia Nacional de Promoción Científica y Técnica, the CONICET and the Howard Hughes Medical Institute (HHMI).

Note added in proof

During the production process of this review an article has been published (Duan, Q., Kita, D., Johnson, E.A., Aggarwal, M., Gates L., Wu H-M. and Cheung A.Y. (2014) Reactive oxygen species mediate pollen tube rupture to release sperm for fertilization in Arabidopsis. Nat Commun. 5:3129. doi: 10.1038/ncomms4129) which is in agreement with our previous observations about the role of ROS during fertilization.

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