Plant Signaling & Behavior

ISSN: (Print) 1559-2324 (Online) Journal homepage: http://www.tandfonline.com/loi/kpsb20

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To cite this article: María Victoria Martín, Ayelén Mariana Distefano, Eduardo Julián Zabaleta & Gabriela Carolina Pagnussat (2013) New insights into the functional roles of reactive oxygen species during embryo sac development and fertilization in Arabidopsis thaliana, Plant Signaling & Behavior, 8:10, e25714, DOI: 10.4161/psb.25714

To link to this article: http://dx.doi.org/10.4161/psb.25714

Published online: 18 Jul 2013.

Plant Signaling & Behavior 8:10, e25714; September 2013; © 2013 Landes Bioscience

New insights into the functional roles of reactive oxygen species during embryo sac development and fertilization in *Arabidopsis thaliana*

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Keywords: reactive oxygen species, gametogenesis, embryo sac, female gametophyte, pollination, plant reproduction

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; MnSOD, manganese SOD; H_2O_2, hydrogen peroxide; O^{2-}, superoxide anion; PCD, programmed cell death

Previously considered as toxic by-products of aerobic metabolism, reactive oxygen species (ROS) are emerging as essential signaling molecules in eukaryotes. Recent evidence showed that maintenance of ROS homeostasis during female gametophyte development is crucial for embryo sac patterning and fertilization. Although ROS are exclusively detected in the central cell of mature embryo sacs, the study of mutants deficient in ROS homeostasis suggests that controlled oxidative bursts might take place earlier during gametophyte development. Also, a ROS burst that depends on pollination takes place inside the embryo sac. This oxidative response might be required for pollen tube growth arrest and for sperm cell release. In this mini-review, we will focus on new insights into the role of ROS during female gametophyte development and fertilization. Special focus will be made on the mitochondrial Mn-Superoxide dismutase (MSD1), which has been recently reported to be essential for maintaining ROS homeostasis during embryo sac formation.

The Apparent Paradox of ROS

As aerobic organisms, plants live in an oxygen rich environment. Inevitable, reactive oxygen species (ROS) by-products and their chemical reactions are part of their basic metabolism. Initially documented as a toxic consequence of aerobic metabolism, ROS can also work as signaling molecules regulating crucial developmental and physiological events in many different organisms. Plants, animals, and even fungi have evolved mechanisms in which ROS are used as messengers to fulfill an extensive range of key biological processes. In *Drosophila melanogaster*, a signaling role for ROS was demonstrated in the differentiation of common myeloid progenitors in hematopoietic cells.[1] In vertebrates, ROS production by NADPH oxidase (NOX) enzymes are involved in neuronal maturation and differentiation during brain development,[2] cell migration[3] and in stem cell self-renewal and differentiation.[4] ROS also play a direct role in sexual development, hyphal growth and cytoskeleton organization in fungi.[5-7] In plants, growing evidence supports a role for ROS in an extensive range of environmental responses and developmental processes such as response to biotic and abiotic stresses, hormonal signaling, division, cell elongation, root development, apical dominance, tracheary element maturation, trichome development, senescence and programmed cell death.[8-18] This apparent biological paradox is based on the fact that ROS could work either as toxic agents or as regulating biomolecules depending basically on concentration and pulse duration. The fine-tuned balance between ROS production and scavenging/antioxidants is thus crucial for normal growth and development. Accordingly, ROS oscillations and homeostasis are tightly regulated in the cell. In plants, a complex network of ROS production and scavenging operates in all subcellular compartments to overcome ROS toxicity.[19] Additionally, the same antioxidant mechanism seems to allow the use of ROS as signaling molecules.

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Submitted: 07/02/13; Accepted: 07/10/13
Citation: Martín M, Distefano A, Zabaleta E, Pagnussat G. New insights into the functional roles of reactive oxygen species during embryo sac development and fertilization in Arabidopsis thaliana. Plant Signaling & Behavior 2013; 8:e25714; http://dx.doi.org/10.4161/psb.25714

Female Gametogenesis and ROS

Plants differentiate their gametes inside specific haploid structures called gametophytes. The male gametophyte is the pollen grain, while the female gametophyte is the embryo sac, also called megagametophyte. The embryo sac is a 7-cell structure that includes 4 different cell types: 2 gametic cells, the egg cell and the central cell, 2 synergid cells and 3 antipodal cells. The egg cell is located at the micropylar edge of the embryo sac and is flanked by 2 synergid cells. Synergid cells are responsible for pollen tube attraction and reception and together with the egg cell form what is called the egg apparatus.[20,21] Most of the female gametophyte is occupied by a large central cell. The central cell is the only cell in the embryo sac that is diploid, as it nucleus results from the fusion of two haploid polar nuclei before fertilization. Three antipodal cells are located at the chalazal end of the embryo sac. Their function is still unclear and they appear to undergo programmed cell death right after fertilization. The whole gametophytic structure

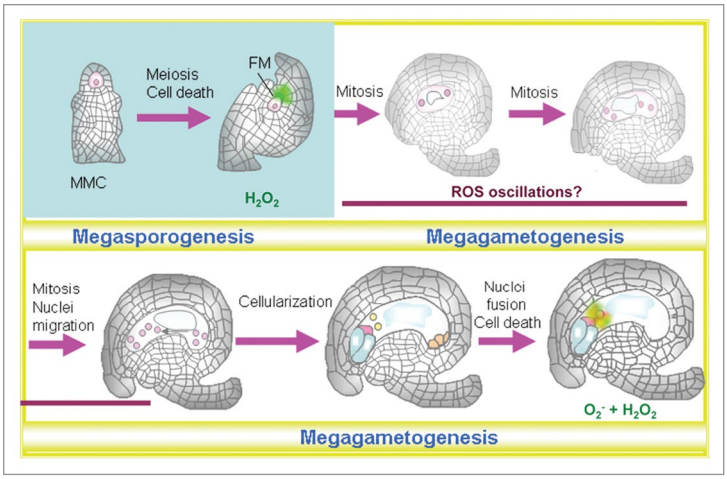

Figure 1. Illustration showing ROS domains during megasporogenesis and female gametogenesis in *Arabidopsis. thaliana*. During megasporogenesis, an oxidative domain that corresponds to cytosolic peroxide is detected in the nucellus of the developing ovule. The location corresponds to the position of the dying megaspores. ROS cannot be detected during early megagametogenesis, suggesting a tight control of the embryo sac oxidative status. Accordingly, high expression of *MSD1* is detected. ROS oscillations might control mitosis progression as reported for other systems. At maturity, mitochondrial superoxide and cytosolic peroxide are detected exclusively in the central cell of the embryo sac. MMC, megaspore mother cell; FM, functional megaspore.

originates from a haploid megaspore that differentiates inside the ovule primordium. Three successive nuclear division cycles originate a syncytium of eight nuclei. Cellularization and further differentiation take place to outline the seven celled structure of the embryo sac. ROS are first detected during megaspores cell death (**Fig. 1**). A strong burst of peroxide takes place at the nucellus, in a position that corresponds with the dying megaspores. In agreement, hydrogen peroxide (H_2O_2) has been recognized as key modulators of PCD in plants.[15] In developing embryo sacs, ROS cannot be detected using common probes or staining. However, insertional female gametophytic mutants impaired in MnSOD activity-named *oiwa* mutants- that showed abnormal high levels of ROS presented mitotic arrest during megagametogenesis. Mutant embryo sacs are arrested at FG3 or FG4 stages, in which only 2 or 4 nuclei respectively are present inside the embryo sac.[22] The elevation of ROS concentration detected inside the mutant gametophytes might interfere with the mitotic machinery. ROS were reported to regulate cell cycle progression, microtubule organization, and cell plate formation through the modulation of key components of the cell division machinery. Additionally, ROS were reported to regulate Ca^{2+}gradients and proteins such as cyclin dependent kinases, MAPs, and possibly aurora kinases.[23,24] Such regulation of mitotic division inside WT embryo sac cannot be discarded. Intracellular ROS oscillations that are probably not detectable by traditional techniques might also regulate mitosis during female gametogenesis. *oiwa* gametophytes present aberrant nuclei migration along the embryo sac, suggesting that ROS might also disturb microtubule organization.[22] In agreement with this idea, the formation of atypical tubulin paracrystals has been reported to occur under elevated ROS levels via MAPK activation and MAP65 phosphorylation.[24] As cell fate inside the embryo sac seems to rely on positional information, abnormal nuclei migration during development has been associated with anomalous differentiation of cells inside the gametophyte.[25-27] Thus, ROS homeostasis appears essential not only for normal nuclei division and migration, two crucial events that characterize early megagametogenesis, but also to guarantee a properly patterning of the embryo sac.

In the mature female gametophyte, peroxide and mitochondrial superoxide are detected exclusively in the central cell.[22] Notably, ROS are not observed around the antipodal cells, which have been showed to undergo cell death in mature embryo sacs.[28] However, as the central cell was reported to regulate antipodal cells lifespan, the accumulation of ROS in the central cell and more specifically inside central cell mitochondria, might work as a signal to generate antipodal cell death in a non-cell autonomous way.[22,28]

The particular location of ROS in mature embryo sac is also interesting. The central cell plays critical roles during female gametophyte development. It regulates not only antipodal cell degeneration but also pollen tube guidance and the initiation of endosperm after fertilization.[29] Additionally, the central cell is characterized by a global demethylation. This hypomethylation in the central cell is thought to cause transposable element transcription and generation of 24 nt small interfering RNAs (siRNAs). These siRNAs might migrate to the egg cell, where they guide de novo DNA methylation.[30-32] Therefore, the oxidative status of the central cell might be important not only for regulating developmental aspects of the gametophyte but also for directing DNA methylation, as has been reported for other systems.[33,34]

MSD1 and Female Gametophyte Development

Superoxide dismutases (SODs; EC 1.15.1.1) catalyze the dismutation of superoxide to molecular oxygen and peroxide (H_2O_2) and constitute the first line of cellular defense against ROS.[35,36] The majority of plants contain a number of SOD isozymes that are classified according to their metal cofactors into 3 types: iron SOD (FeSOD), manganese SOD (MnSOD), and copper–zinc SOD (Cu/ZnSOD). Three FeSOD encoding genes (*FSD1*, *FSD2*, and *FSD3*), 3 Cu/ZnSOD encoding genes (*CSD1, CSD2,* and *CSD3*) and 1 MnSOD encoding gene (*MSD1*) can be found in the *Arabidopsis thaliana* genome.[37] Plant MnSODs are highly conserved and their role in tolerance to a variety of environmental stresses has been extensively studied.[15,38-40] Although MSD1 was proposed to be a key component of the ROS regulatory network, there have been relatively few studies that have focused on its role in regulating ROS homeostasis during development.

Arabidopsis antisense lines with decreased *MSD1* expression showed retarded root growth,[41] and a high throughput forward genetic screen to find gametophytic mutants in *Arabidopsis* identified a transposon insertion line in MSD1 (named *mee33* for "maternal effect embryo arrest 33" that was classified as a female gametophytic mutant, as it showed defects in female transmission.[42] More recently, *MSD1* expression was detected during megasporogenesis and female gametophyte development in

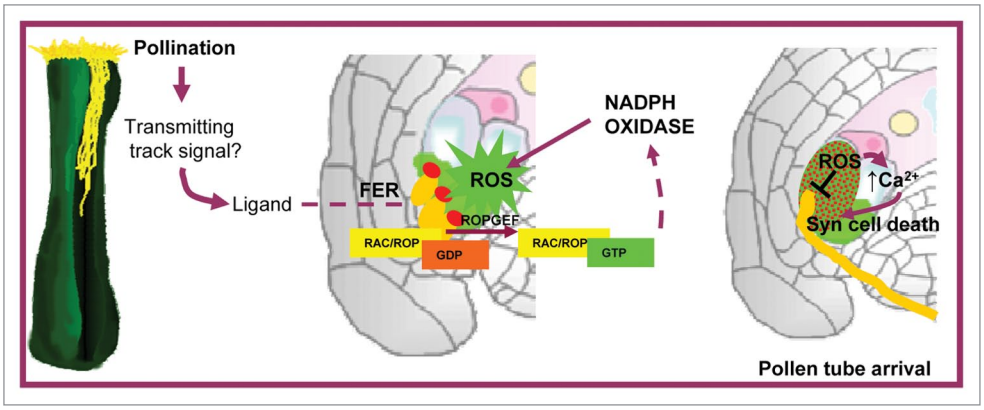

Figure 2. ROS-mediated signaling pathways proposed to control pollen tube growth arrest/burst in *A. thaliana*. Pollination triggers an oxidative burst in the synergid cells inside the unfertilized embryo sac. This oxidative environment induced by the accumulation of mitochondrial superoxide and cytosolic peroxide is restricted to the synergid cells and maintained until pollen tube arrival. ROS production inside the embryo sac might be dependent on FER signaling. As described for root hairs, FER might interact with ROPGEF which in turn interacts and activates RAC/ROP. Downstream signaling might lead to the activation of NADPH oxidase to produce ROS. ROS increase inside the receptive synergid cell might be necessary for pollen tube growth arrest or burst. Additionally, it might lead to an influx of Ca^{2+} from the extracellular space. The increase in Ca^{2+} concentration could in turn promote synergid cell death.

Arabidopsis.[22] *MSD1* expression seems to be strongly regulated during female reproductive development and shows a complementary pattern with the distribution of ROS, particularly mitochondrial superoxide.

During megasporogenesis, a strong oxidative burst that corresponds to peroxide is detected around the dying megaspores. No mitochondrial superoxide can be observed, which is in agreement with the high levels of expression shown by *MSD1* in the nucleus at that stage.[22] *MSD1* expression can be detected along the whole embryo sac during female gametophyte development. As gametogenesis advances, *MSD1* expression becomes restricted to the egg apparatus cells, and it is not detectable in the central cell before pollination. Again, ROS show a complementary pattern in WT embryo sacs, as they cannot be detected in the egg apparatus but are detectable in the central cell of mature female gametophytes.[22] Recent studies of 2 independent T-DNA insertion lines allelic to MEE33 (*oiwa-1* and *oiwa-2* mutants) revealed a role for MSD1 as an essential protein regulating ROS levels during female gametogenesis. In *oiwa* embryo sacs, ROS homeostasis is disturbed, presenting high levels of peroxide and mitochondrial superoxide all along the female gametophyte. High ROS inside the embryo sacs results in infertility or arrested embryogenesis.[22] Besides mitotic arrest and abnormal nuclei migration, *oiwa* embryo sacs showed gametophytes in which egg apparatus cells seem to acquire central cell features, expressing central cell-specific markers. As the high levels of ROS detected in *oiwa* mutant embryo sacs precede gametophytic cell specification, it is possible that ROS might function as signaling molecules determining central cell fate. Thus, *MSD1* expression in the embryo sac appears essential to restrict central cell specification and to regulate ROS homeostasis, which is critical for key aspects of embryo sac development, such as cell division progression, nuclei migration, and cell fate decisions.[22] Moreover, not only mitochondrial superoxide is deregulated in *oiwa* embryo sac. Cytosolic superoxide levels are also increased in the mutants, suggesting that other sources of ROS, such as NADPH oxidase activity, might be also involved, probably as a consequence of mitochondrial dysfunction. Therefore, MSD1 appears to be essential to maintain cellular ROS homeostasis in the embryo sac at specific domains and stages of development.

ROS and Embryo Sac Fertilization

Once pollen germinates in the stigma, pollen tubes grow through many different maternal tissues to reach the fully developed embryo sacs. Chemoattractants located in the pistil are thought to guide pollen tubes toward the ovules.[43-45] Recent reports showed that female gametophytes respond to stigma pollination or pollen tube growth through the female transmitting track, supporting the existence of long distance signaling pathways. The expression of the *AMC* gene, named after the *abstinence by mutual consent* mutant, encodes a peroxin required for protein import into peroxisomes and essential for pollen tube discharge, is induced in the embryo sac by pollen deposition on the stigma.[46] Additionally, pollination triggers an oxidative burst in the synergid cells inside mature unfertilized female gametophytes.[22] This oxidative environment inside the embryo sac is restricted to the synergid cells and is maintained until pollen tube arrival. 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 regulation of ROS levels from the maternal side is crucial for embryo development.[22]

Micropylar pollen tube attraction toward the female gametophytes is regulated by synergid cells and the central cell, as has been demonstrated by the use of a wide number of *Arabidopsis* mutants and research in *Tourrenia fournieri*.[20,47-49] LUREs, a group cysteine-rich proteins (CRPs) belonging to the defensin-like proteins subgroup were first shown to attract pollen tubes in *Tourrenia fournieri*, where are secreted from the micropylar end of the female gametophyte.[20] LUREs have been also identified in *Arabidopsis* as

pollen tube attractants guiding pollen tubes toward the ovules.[50] Once the pollen tube arrives to the micropyle of the ovule it continues to grow and enters one of the synergid cells.[51] Initial communication between the pollen tube and the receptive synergid was proposed to occur via the activation of a receptor kinase called FERONIA (FER) at the synergid's surface.[52] In *fer* mutants, pollen tubes are attracted to the embryo sacs, but instead of arresting and delivering the sperm cells at the receptive synergid, they continue to grow invading the embryo sac. In root hairs, FER interacts with the plant Rho small GTPase (RAC/ROPs). Experiments with *fer* mutants and plants overexpressing RAC/ROPs or FER indicate that the interaction controls NADPH oxidase-dependent ROS production.[53] Production of ROS stimulate Ca^{2+} channels increasing cytoplasmic Ca^{2+} concentration, which in turns promotes root hair growth.[53] A similar pathway might be activated in the female gametophyte in response to pollination (**Fig. 2**). Not only ROS are detected in the synergid cells after pollination. Recently, cytoplasmic Ca^{2+} oscillation have been detected in synergid cells upon pollen tube arrival.[54] The role of these oscillations is still unclear, but they might be involved in the induction of the receptive synergid cell death that follows pollen tube burst. Likewise, pathogen-associated Ca^{2+} elevation in plant cells activates a signal transduction pathway that leads to hypersensitive response and cell death.[55] Further studies in the *fer* background would be helpful to shed light on the mechanisms that depend on FER activity and to determine if ROS production in the synergid cells depend on FER as was demonstrated in root hairs. Furthermore, detailed studies will be needed to determine if ROS are required for pollen tube attraction, growth arrest or pollen tube burst.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Dr Frantisek Baluska for kindly inviting this review. MVM. is a postdoctoral fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). EJZ and GCP are CONICET researchers. The research in Dr Pagnussat's lab was funded by Agencia Nacional de Promoción Científica y Técnica, CONICET and Howard Hughes Medical Institute (HHMI). We thank members of the mitochondrial biology lab for helpful discussions and suggestions.

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