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POLLEN: AN AMAZING MODEL SYSTEM TO STUDY A DISPARATE SERIES OF PLANT BIOLOGICAL PROCESSES

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Abstract

Pollen, the male gametophyte of higher plants, represents an extremely interesting, and peculiar, experimental system for the study of diverse aspects of plant biology, apart from the - obvious - research on gametophytic development in itself. For example, cell cycle progression, as the formation of mature pollen from the microspore mother cells consists in a series of specific, often synchronous cell divisions: meiosis, the asymmetric microspore mitosis and the division of the generative pollen cell. Also, for the study of the interactions between the gametophyte and the sporophyte during pollen development, the formation of the - unusual - pollen wall or intracellular transport processes during pollen tube growth. Instead of dedifferentiating and forming a callus, as normally happens with any isolated plant cell, microspores isolated from the anthers can continue in vitro their normal developmental programme, giving rise to mature, functional pollen. However, triggered by specific stress treatments, microspores and immature pollen grains can switch towards sporophytic development with the formation of (haploid) embryos and plants. These and other unique characteristics of the pollen system will be discussed in this review.

Key words*: cell cycle, gametophytic development, microspore embryogenesis, pollen tube, pollen wall.*

INTRODUCTION: DEVELOPMENT OF THE MALE GAMETOPHYTE IN HIGHER PLANTS

Sexual plant reproduction success relies on the synchronised production of viable male and female gametic cells. Pollen, the male gametophyte of higher plants, carries the genetic information of the male parent and brings it to the female gametophyte, the embryo sac, to achieve fertilisation (Ma and Sundaresan, 2010). Pollen represents the (male) gametophytic generation in the life cycle of angiosperms, which has been extremely reduced during evolution, in relation to the sporophytic generation. The 'life span' of the male gametophyte, from the sporophytic precursors to mature pollen is, generally, very short, of only a few days. Moreover, mature pollen is, apparently, a very simple structure, with only two or three cells, depending on the species. Male gametophytes in angiosperms are formed within the anthers - the male sexual organs - passing through two sequential processes, microsporogenesis and microgametogenesis. Each diploid precursor, a microsporocyte or pollen mother cell, gives rise to four haploid, immature microspores through meiosis, a highly synchronised process, which represents the transition from the sporophytic to the gametophytic generation and the beginning of microsporogenesis. Initially, after the two meiotic divisions, the four microspores are kept together, in a structure called tetrad, by a callose external layer. Upon digestion by callase, an enzyme secreted by the tapetum - the anther wall tissue surrounding the anther locus - the microspores are released from the tetrads. Microspore maturation involves an increase in size, several cytological changes, acquisition of an ellipsoidal shape and the formation of a big vacuole pushing the nucleus to one of the cell poles. Microgametogenesis is initiated by the division of the mature, uninucleate microspore, through an asymmetric mitosis (pollen mitosis I, PMI), the result of which is a peculiar bicellular structure, with the generative cell included in the cytoplasm of the larger vegetative cell. Immature bicellular pollen will suffer further cytological and metabolic changes, such as amylogenesis, amylolysis or carbohydrate interconversion, giving rise to the mature gametophyte (Clément and Pacini, 2001; Hafidh et al., 2016; Carrizo-

García et al., 2017; Liu and Wang, 2021). Figure 1 shows some of these developmental stages during maturation of tobacco pollen. The last phase of pollen development in the anther, just before anthesis, involves a strong dehydration of the pollen grain and its developmental arrest (Pacini and Dolferus, 2019), necessary for pollen to survive as a 'free-living' organism - a unique example for a plant cell - until landing on the stigma. The generative cell must undergo a second, symmetrical mitosis (pollen mitosis II, PMII) to generate the two sperm cells (the male gametes). PMII can occur either during pollen maturation in the anther, in plant species with tricellular pollen, or within the pollen tube during germination through the style, in bicellular pollen species.

Interestingly, the highly regulated process of *in vivo* pollen development can be mimic in *in vitro* culture systems - at least in the model tobacco (Benito Moreno et al., 1988) and some other species. Uninucleate microspores isolated from the anthers after they are released from the tetrads, can continue *in vitro* their normal developmental programme in a simple culture medium, giving rise to mature, functional pollen grains. However, specific stress treatments applied to the same isolated microspores (or to immature bicellular pollen grains) arrest normal gametophytic development and activate an alternative sporophytic programme, inducing the formation of 'embryogenic pollen', from which haploid embryos and plants can be generated. This process, known as androgenesis, is highly interesting, both for basic studies on cell differentiation and for the generation of homozygous double haploid lines for plant breeding programmes (Touraev et al., 1996a; Testillano, 2019).

These and other specific characteristics make pollen unique amongst plant cells, and an extremely interesting experimental system for the study of diverse aspects of plant biology. First, obviously, research on gametophytic development in itself, and the interactions between the gametophyte and the sporophyte during pollen development *in vivo*. But also, on cell cycle progression, the effects of stress on cell differentiation and specific developmental processes, intracellular transport during formation of the (amazing) pollen tube, or the (also unique) structure of the pollen wall. Some examples of the use of microspores or pollen as a research tool and relevant advances in the areas mentioned above, are briefly described in the following sections.

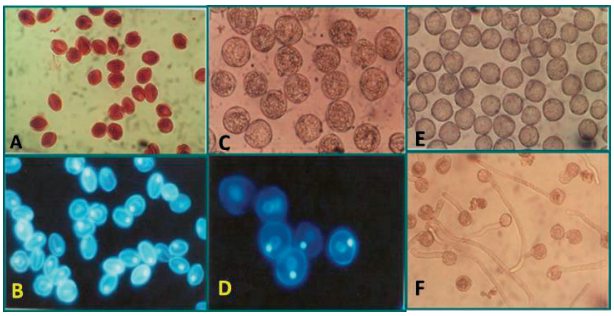

Figure 1. Pollen development in tobacco. Microspores and pollen, at different developmental stages, observed under the light microscope (A, C, E, F) or the fluorescence microscope after staining with DAPI (B, D). Late uninucleate microspores (A, B); in B, observe, in most cells, the nucleus, with condensed chromatin, in one of the poles of the ellipsoidal-shaped microspore. Mid-bicellular pollen (C, D); in D, observe the highly condensed generative nucleus within the vegetative cell. Mature pollen (E). Pollen germinating in a standard *in vitro* germination medium (F). (A. Touraev, E. Heberle-Bors, O. Vicente, original images)

CELL CYCLE REGULATION

A cell cycle comprises the period from the generation of a new cell, to completing its division with formation of the two daughter cells; it is divided into four successive major phases, named Gap 1 (G1), Synthesis (S), Gap 2 (G2), and cell division (M) (Inzé and De Veylder, 2006). Depending on the final cells produced, specifically on its DNA content, two types of cell divisions are considered. First, a meiotic or reductional division, which generates as the final product daughter cells with half DNA content and is limited to gametes production. Second, a mitotic division, in which both daughter cells have the same DNA content as the mother cell. Moreover, a mitotic division can be symmetric, generating two identical cells, or asymmetric, producing two daughter cells of different size and, generally, different structural features, gene expression patterns and - most important - different developmental fates. An asymmetric division often represents the initiation of a cell differentiation process (De Smet and Beeckman, 2011).

Along pollen development, all types of cell division mentioned above take place in a sequential manner. The two meiotic divisions of a microspore mother cell produce four haploid microspores, in the G1 phase of the cell cycle. During microspore maturation, DNA replication takes place (S phase), so that the mature vacuolated microspore is in the G2 phase, ready to undergo PMI, the asymmetric mitotic division that generates (immature) bicellular pollen. As mentioned above, bicellular pollen has the peculiar structure of 'a cell (generative)-within-a cell (vegetative)'. Immediately after mitosis, the generative cell enters S-phase, replicating its DNA, and remains in the G2-phase until PMII. The vegetative cell, on the other hand, does not proceed through a new cell cycle and is arrested in G1 (G0 state) (Zarsky et al., 1992).

Microspores constitute an ideal experimental system to study the effect of mutations on cell cycle progression, as they represent a single cell lineage and because of its haploid nature (Li et al., 2015). In fact, several genes specific for gametophytic developmental processes and affecting both, meiosis and mitosis, have been characterised, mostly in the model species *Arabidopsis thaliana*. For example, Solo Dancers (SDS) encodes a meiotic-specific cyclin that interacts with cyclin-dependent kinases (CDKs); in *Arabidopsis*, mutation of this gene causes random chromosome distribution and leads to abnormal meiotic products (Azumi et al., 2002). A more complete description of the control of cell cycle progression during meiosis, based on CDK activities, can be found in Cromer et al. (2012).

Cell cycle regulation during gametogenesis is also essential to finally produce functional gametes. DUO POLLEN 1 (DUO1) and DUO2 play sequential regulatory roles during mitosis of the generative cell (PMII); *duo1* mutants fail to enter mitosis at the G2/M transition, whereas *duo2* mutants enter pollen mitosis II, but are arrested at prometaphase (Durbarry et al., 2005). Later-on, the conserved protein DUO3 was shown to be involved in the control of cell cycle progression, and its role was independent of the G2/M regulator CYCB1;1 (Brownfield et al., 2009). Also, E3-mediated ubiquitination of repressors are important checkpoints for cell cycle progression (Inzé and De Veylder, 2006). Specifically, during gametogenesis the E3 ubiquitin ligase RHF1a interacts and targets for degradation the cyclin-dependent kinase inhibitor ICK/KRP6; inhibition of this process in *rhf1a rhf2a* double mutants causes microspore arrest in interphase (Liu et al., 2008). Similarly, loss of function of the F-box protein FBL17 blocks progression through PMII, the mitosis of the generative cells, so that the two sperm cells are not formed (Gusti et al., 2009). Finally, the APD2 protein has shown E3 ligase activity *in vitro* and, in a redundant way to its related proteins APD1, APD3 and APD4, is critical for the progression of PMII during male gametophyte development (Luo et al., 2012).

The study of both, male and female gametophyte development has largely contributed to elucidate the molecular machinery of epigenetic regulation during cell cycle progression. Transcriptomic analysis of meiocytes showed that enzymes related to DNA methylation, chromatin modification and small non-coding RNAs are differentially expressed during gametogenesis, suggesting the involvement of these mechanisms in the regulation of both, meiotic and post-meiotic gametophytic development (Chen et al., 2010; Schmidt et al., 2011). Many later reports have confirmed and extended these early studies (see Ashapkin et al., 2019, for a recent review).

IN VITRO MICROSPORE MATURATION

Any isolated plant cell, when cultured *in vitro*, dedifferentiate forming a callus. Microspores represent a remarkable exception to this general behaviour. Microspores, isolated from the anthers after release from the tetrads, at the mid-uninucleate or later developmental stages (including immature bicellular pollen), can continue *in vitro* their normal gametophytic developmental programme under specific experimental conditions, giving rise to mature, functional pollen grains. Efficient *in vitro* microspore maturation systems have been established in a few plant species such as tobacco, *Antirrhinum majus*, or canola (Benito Moreno et al., 1988; Barinova et al., 2002; Sheoran et al., 2009).

In tobacco, isolated microspores mature *in vitro* by incubation at 25°C in a simple liquid

medium containing mineral salts, sucrose as a carbon source, and metabolic precursors: amino acids (as a protein hydrolysate) and nucleosides (Benito Moreno et al., 1988; Tupy et al., 1991), but without the addition of phytohormones, protein factors or other components. Pollen development *in vivo* is completely dependent on the sporophytic tissues of the anther, specifically on the tapetum. However, from the mid-uninucleate stage onwards, the essential function of the tapetum appears to be providing energy, nutrients and metabolic precursors to the developing microspores and pollen, even though other tapetum-derived compounds may enhance the gametophytic function of the mature pollen. Otherwise, the pollen developmental programme, once microspores are individual cells within the anther locus, seems to be independent of the sporophyte. This might be also the reason why maturation of isolated microspores is feasible in *in vitro* cultures.

The *in vitro* maturation cultures are useful for the study of male gametophytic development without interference of the anther tissues, and the interactions between the gametophyte and the sporophyte during this process (see below).

INTERACTIONS BETWEEN THE GAMETOPHYTE AND THE SPOROPHYTE DURING POLLEN DEVELOPMENT

The gametophytic generation in angiosperms can be considered, in a way, as an endosymbiont of the sporophyte: haploid cells that develop within a diploid organism, with a short period as independent individuals (in the case of the male gametophyte) upon their release from the anther at anthesis (Hafidh et al., 2016). During gametophytic development, microspores are surrounded by the sporophytic tapetum tissue, which plays several essential roles in this process: i) contributes to microspore release from the tetrads, secreting callase, ii) provides energy and nutrients to the developing microspores, and iii) tapetum degradation actively participates in pollen cell wall formation and sporopollenin deposition on the exine (Mariani et al., 1990; Pacini et al., 1985; Scott et al., 2004). The relevant function of the tapetum on pollen development is supported by the fact that plants with disrupted tapetum result in nuclear male sterility (Mariani et al., 1990), and the analysis of male sterile mutants affected on meiosis revealed that, in some cases, the activity of the tapetum was altered (McCormick, 2004; Scott et al., 2004).

At the initiation of microsporogenesis, callose surrounds pollen mother cells and isolates them from the anther tissues, which is a critical mechanism to achieve meiosis. The accumulation of callose continues throughout meiosis, and once microspores are completely developed, callose is degraded and microspores are individually released into the pollen sac (Wang et al., 2020; Wan et al., 2011). Both, the biosynthesis and degradation of callose during microsporogenesis is controlled by the sporophyte at the tapetum level (Ünal et al., 2013).

Interaction between the sporophyte and the gametophyte is also evident in more advanced developmental steps, during gametogenesis. Formation of the outer pollen layer, the exine, and sporopollenin deposition, for instance, are controlled by the sporophytic tissue (Paxson-Sowders et al., 2001; Li et al., 2017; Suzuky et al., 2017). The pollen wall, therefore, includes material directly derived from the tapetum (Blackmore et al., 2007). Moreover, the tapetum provides developing microspores compounds of different nature, such as pectins, proteins or lipid bodies, which may contribute to stimulate pollen development (Loubert-Hudon et al., 2020). Also, some secondary metabolites, such as flavonoids, are synthesised in the anther and accumulate in the mature pollen wall, enhancing pollen development and pollen function by different mechanisms (Ylstra et al., 1992; van Eldik et al., 1997; Thomson et al., 2010; Li et al., 2012; Ning et al., 2018; Zhang et al., 2020).

However, as discussed above, isolated microspores of different species can develop under specific *in vitro* conditions to form functional mature pollen. This indicates that, after microspore release from the tetrads, the tapetum is somehow dispensable, as far a source of energy, nutrients, and metabolic precursors, provided *in viv*o by the anther, are included in the culture medium.

THE POLLEN WALL

Plant cell walls are essential as protecting barriers and for maintaining cell shape, but also play important roles in cell-to-cell communication. In microspores and pollen, cell wall composition and characteristics are dynamic and change throughout development; moreover, they possess some specific features, not present in other plant cell walls (Blackmore et al., 2007). Immature microspores present a cellulose-rich envelope upon callose degradation and initiation of programmed cell death in the tapetum (Shi et al., 2015a). This initial cell wall, or primexine, serves as substrate for the deposition of sporopollenin, one of the strongest biopolymers found so far in nature and, interestingly, present exclusively in plant spores and pollen cell walls amongst plant cells. Moreover, due to its physical characteristics, sporopollenin has applications in different areas, such as solid support for peptide synthesis, catalysis, and ion-exchange chromatography (Mackenzie et al., 2015).

Mature pollen grains show a multilayered envelope referred to as sporoderm. The inner layer, or intine, is located just above the plasma membrane and has similar composition and features as somatic cell walls (Li et al., 1995). The outer pollen wall, the exine, is the most complex and diverse plant cell wall structure reported so far (Blackmore et al., 2007). A specific feature of the exine is the presence of callose, which is more rapidly metabolised than other cell wall polysaccharides such as cellulose (Liu and Wang, 2021). Callose accumulation in the cell walls seems to play an essential regulatory role during the early stages of pollen development, around meiosis, when callose is an abundant component; in later stages, during microgametogenesis, callose is replaced by other polysaccharides, such as cellulose (Blackmore et al., 2007; Liu and Wang, 2021). Mutations affecting callose metabolism result in a thin or even absent callose-enriched wall (Shi et al., 2015b) that, in some cases, can affect proper pollen maturation (Li et al., 2020), and therefore, compromise male fertility. Finally, mature pollen cells have a special lipidic layer called tryphine or pollen coat that is disposed in the outer side, filling the gaps of the exine (Rejón et al., 2016). The pollen coat plays an important role in the interactions of pollen with pollinators and the stigma.

THE POLLEN TUBE

Once a pollen grain reaches the stigma of the pistil, pollen is re-hydrated and a cell-to-cell recognition takes place, triggering pollen germination and the formation of the pollen tube. Its function is to deliver the two sperm cells - already present in the mature pollen grain before anthesis, or generated by the division of the generative cell during pollen tube growth within the style - to the female gametophyte, the embryo sac, to achieve the double fertilisation characteristic of higher plants (Leydon et al., 2014). The extraordinary nature of the pollen tube becomes evident if we consider the average diameter of a pollen grain (30-50 μm) and the length of the style, highly variable but reaching, in some flowers, more than 10 cm. This means that a single cell (the vegetative pollen cell) generates in a short time, generally only a few hours, a structure that can be more than 2000 times longer than the cell diameter.

Pollen tube development and growth constitutes an excellent system to study several cytological processes, such as vesicle trafficking (Ruan et al., 2021), dynamic coordination between endocytosis and exocytosis (Guo and Yang, 2020), cytoskeleton dynamics (Fu, 2015), as well as molecular signalling processes that take place specifically at the plasma membrane level. The latter include those mediated by Ca^{2+}, ion transport and pH homeostasis (Michard et al., 2017; Li et al., 2021), phospholipids (Vaz Dias et al., 2019), and ROP-type Rho GTPases activity.

A very special feature of pollen tube growth is its capability to receive and respond very quickly to many exogenous signals from the female tissue in the pistil. A key point of this developmental plasticity is the pollen tube cell wall dynamics and composition, which provide special features to mediate the communication between inner and outer sides of cells (Ringli, 2010). This communication and developmental versatility of the pollen cell wall relies on cell surface and/or transmembrane receptors. Upon perception of specific stimuli, individual

responses are triggered through MAP kinase-mediated signalling cascades (Levin, 2005). Moreover, pollen tube cell wall has a particular chemical composition, consisting on irregularly distributed pectins, callose, cellulose, and hemicelluloses, amongst others, along its surface (Dardelle et al., 2010; Guo and Yang, 2020). This specific composition determines the physical characteristics of the pollen tube, and ts final shape in response to external physical forces.

Altogether, the possibility to follow pollen tube growth, both *in viv*o and in *in vitro* germination cultures, and the relatively easy manipulation of this process, makes it one of the most popular cell model systems to study the mechanisms behind polar cell expansion (Guo and Yang, 2020).

ANDROGENESIS

The tightly regulated programme of gametophytic pollen development can be switched *in vitro*, under specific stress conditions, towards a sporophytic pathway, producing haploid embryos and plants from microspores or immature bicellular pollen, in a process known as 'androgenesis'. This possibility is known since the 1960s, through the pioneering experiments of Guha and Maheshwari (1964) in anther cultures of *Datura innoxia*. Nowadays, the generation of haploid plants through anther culture has been established in hundreds of species. There are also several efficient *in vitro* systems of androgenesis from isolated microspores or immature pollen grains. For example, in tobacco (Touraev et al., 1996a) or wheat (Touraev et al., 1996b).

Microspore embryogenesis is a useful biotechnological tool to generate in one generation homozygous double haploid (DH) lines for plant breeding programmes (Foster et al., 2007). The embryogenic response depends to a large extent on the species, or even the specific genotype within a species. Microspores isolated form rapeseed (*Brassica napus*) and barley (*Hordeum vulgare*) are, in general, highly embryogenic and are used as model species for dicotyledonous (in addition to tobacco) and monocotyledonous plants, respectively, to better understand the biological processes behind androgenesis induction (Seguí-Simarro, 2016; Pérez-Pérez et al., 2019a). Other species, such as tomato (*Solanum lycopersicum*) or *Arabidopsis thaliana*, seem to be recalcitrant to this process. However, the absence of successful microspore embryogenesis in these species may be due to the lack of effective induction protocols developed so far.

On the other hand, the nature of the stress treatment applied to activate the sporophytic developmental pathway is species-dependent. Microspores belonging to different species respond to either heat, cold, starvation, changes in pH, oxidative stress, osmotic stress, chemical treatments or, in some cases, a combination of some of the above treatments (Islam and Tuteja, 2012). A critical aspect for androgenesis, which is common to all species tested so far, is the developmental state of microspores. The late, vacuolated microspore stage seems to be the most appropriate for embryogenic induction although, at least in tobacco, somewhat younger microspores and early bicellular pollen are also competent (Touraev et al., 1996c). Finally, some other factors, such as the growing conditions of the donor plant or the *in vitro* culture conditions are critical for androgenic success (Testillano, 2019).

Beside its biotechnological applications, isolated microspore embryogenesis represents an excellent system to study the regulation of cell reprogramming, cell totipotency and cell fate decisions. Furthermore, androgenesis is a useful alternative for the study of the process of embryogenesis itself, due to the technical difficulty to investigate zygotic embryogenesis, where the embryos are surrounded and protected by maternal tissues and are difficult to dissect (Testillano, 2019). Using isolated barley microspores, El-Tantawy et al. (2014) detected low DNA methylation levels upon embryogenic induction, in contrast to the hypermethylated DNA observed during normal gametophyte development. Accordingly, chemical treatments that alter chromatin organisation also showed the importance of this epigenetic regulation in the transition from the gametophytic to the embryogenic pathway in microspores isolated from different species (Li et al., 2014; Solis et al., 2015; Berenguer et al.,

2017; Nowicka et al., 2019). This finding points to isolated microspores as an excellent system to analyse the basis of epigenetic regulation during embryogenesis and/or cell reprogramming.

Moreover, stress applied to isolated microspores causes specific cellular processes such as programmed cell death (PCD; Testillano, 2019), which is barely studied in plants, unlike in animal systems. PCD is a genetically controlled process that takes place upon stress application at the unicellular microspore level and later on, during embryo development (Maraschin et al., 2005). Under inductive conditions, PCD is accompanied by high levels of ROS production (Zur et al., 2009) and autophagy, a mechanism that utilises cells for removing damaged proteins and organelles (Parra-Vega et al., 2015; Pérez-Pérez et al., 2019b; Bárány et al., 2018). Furthermore, stress triggers the synthesis and accumulation of proteolytic enzymes (Berenguer et al., 2017). The activation of all these cellular processes in the microspores, makes this system very useful to study plant responses to stress from a cellular point of view.

Finally, the microspores that respond to the induction stimuli, activating the embryogenetic developmental pathway, acquire specific cytological features. They include the nucleus repositioning to the center of the cell, vacuole fragmentation, reduction in the number of ribosomes and plastids, and mobilisation of starch deposits (Corral-Martínez et al., 2013). Therefore, induction of microspore embryogenesis could be also useful for the study of cellular processes such as organelle rearrangement, movement, ontogenesis and degradation within the cells.

CONCLUSIONS

In this review, we have highlighted some specific (and peculiar) characteristics that make pollen, the male gametophyte of angiosperms, a unique experimental system for the study of diverse cellular and molecular processes in plants. They include, obviously, normal (male) gametophytic development in the anther, but also the formation of the pollen wall - different from the wall of any other plant cell - or the amazing process of pollen tube growth. Pollen development also represents a useful system to investigate cell cycle progression and the interactions of the two generations in the plant life cycle, sporophytic and gametophytic. Microspores, isolated from the anthers after they are released from the tetrads, can continue *in vitro* their normal developmental programme, under appropriate conditions, leading to the formation of mature, functional pollen. However, specific stress treatments block pollen maturation inducing an alternative sporophytic pathway *in vitro*, microspore embryogenesis, which is extremely interesting to study the effect of stress on cell differentiation processes, as well as embryogenesis itself. Moreover, double haploids, generated by anther or isolate microspore cultures, are very useful biotechnological tools in plant breeding. Altogether, the processes and systems described here - pollen development in the anther, microspore maturation and embryogenesis *in vitro* - provide attractive opportunities for basic research in plant biology and practical biotechnological applications.

REFERENCES

Ashapkin, V.V., Kutueva, L.I., Aleksandrushkina, N.I. & Vanyushin, B.F. (2019). Epigenetic Regulation of Plant Gametophyte Development. *International Journal of Molecular Sciences*, *20*(12), 3051.

Azumi, Y., Liu, D., Zhao, D., Li, W., Wang, G., Hu, Y. & Ma, H. (2002). Homolog interaction during meiotic prophase I in Arabidopsis requires the SOLO DANCERS gene encoding a novel cyclin-like protein. *EMBO Journal*, *21*(12), 3081–3095.

Bárány, I., Berenguer, E., Solís, M.T., Pérez-Pérez, Y., Santamaría, M.E., Crespo, J.L., Risueño, M.C., Díaz, I. & Testillano, P.S. (2018). Autophagy is activated and involved in cell death with participation of cathepsins during stress-induced microspore embryogenesis in barley. *Journal of Experimental Botany*, *69*(6), 1387-1402.

Barinova, I., Zhexembekova, M., Barsova, E., Lukyanov, S., Heberle-Bors, E. & Touraev, A. (2002). Antirrhinum majus microspore maturation and transient transformation *in vitro*. *Journal of Experimental Botany*, *53*(371), 1119–1129.

Benito Moreno, R.M., Macke, F., Alwen, A. & Heberle-Bors, E. (1988). In-situ seed production after pollination with in-vitro-matured, isolated pollen. *Planta*, *176*(2), 145–148.

Berenguer, E., Bárány, I., Solís, M.T., Pérez-Pérez, Y., Risueño, M.C. & Testillano, P.S. (2017). Inhibition of histone H3K9 methylation by BIX-01294

promotes stress-induced microspore totipotency and enhances embryogenesis initiation. *Frontiers in Plant Science*, *8*, 1161.

Blackmore, S., Wortley, A.H., Skvarla, J.J. & Rowley, J.R. (2007). Pollen wall development in flowering plants. *New Phytologist*, *174*(3), 483–498.

Brownfield, L., Hafidh, S., Durbarry, A., Khatab, H., Sidorova, A., Doerner, P. & Twell, D. (2009). Arabidopsis DUO POLLEN3 is a key regulator of male germline development and embryogenesis. *Plant Cell*, *21*(7), 1940–1956.

Carrizo-García, C., Nepi, M., & Pacini, E. (2017). It is a matter of timing: asynchrony during pollen development and its consequences on pollen performance in angiosperms - a review. *Protoplasma*, *254*(1), 57–73.

Chen, C., Farmer, A.D., Langley, R.J., Mudge, J., Crow, J.A., May, G.D., Huntley, J., Smith, A.G. & Retzel, E.F. (2010). Meiosis-specific gene discovery in plants: RNA-Seq applied to isolated Arabidopsis male meiocytes. *BMC Plant Biology*, *10*, 280.

Clément, C. & Pacini, E. (2001). Anther plastids in angiosperms. *The Botanical Review*. *67*, 54.

Corral-Martínez, P., Parra-Vega, V. & Seguí-Simarro, J.M. (2013). Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning. *Journal of Experimental Botany*, *64*(10), 3061–3075.

Cromer, L., Heyman, J., Touati, S., Harashima, H., Araou, E., Girard, C., Horlow, C., Wassmann, K., Schnittger, A., De Veylder, L. & Mercier, R. (2012). OSD1 promotes meiotic progression via APC/C inhibition and forms a regulatory network with TDM and CYCA1;2/TAM. *PLoS Genetics*, *8*(7), e1002865.

Dardelle, F., Lehner, A., Ramdani, Y., Bardor, M., Lerouge, P., Driouich, A. & Mollet, J.C. (2010). Biochemical and immunocytological characterizations of *Arabidopsis* pollen tube cell wall. *Plant Physiology*, *153*(4), 1563–1576.

De Smet, I. & Beeckman, T. (2011). Asymmetric cell division in land plants and algae: the driving force for differentiation. *Nature Reviews Molecular Cell Biology*, *12*(3), 177–188.

Durbarry, A., Vizir, I. & Twell, D. (2005). Male germ line development in Arabidopsis. duo pollen mutants reveal gametophytic regulators of generative cell cycle progression. *Plant Physiology*, *137*(1), 297–307.

El-Tantawy, A.A., Solís, M.T., Risueño, M.C. & Testillano, P.S. (2014). Changes in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley. *Cytogenetic and Genome Research*, *143*, 200–208.

Forster, B.P., Heberle-Bors, E., Kasha, K.J. & Touraev, A. (2007). The resurgence of haploids in higher plants. *Trends in Plant Science*, *12*, 368–375.

Fu, Y. (2015). The cytoskeleton in the pollen tube. *Current Opinion on Plant Biology*, *28*, 111–119.

Ruan, H., Jiang, Li., Wang, T. & Ren, H. (2021). Secretory vesicles targeted to plasma membrane during pollen germination and tube growth. *Frontiers in Cell and Developmental Biology*, *8*, 615447.

Guo, J. & Yang, Z. (2020). Exocytosis and endocytosis: coordinating and fine-tuning the polar tip growth domain in pollen tubes. *Journal of Experimental Botany*, *71*(8), 2428–2438.

Guha, S. & Maheshwari, S.C. (1964). *In vitro* production of embryos from anthers of Datura. *Nature*, *204*, 497.

Gusti, A., Baumberger, N., Nowack, M., Pusch, S., Eisler, H., Potuschak, T., De Veylder, L., Schnittger, A. & Genschik, P. (2009). The *Arabidopsis thaliana* F-box protein FBL17 is essential for progression through the second mitosis during pollen development. *PLoS One*, *4*(3), e4780.

Hafidh, S., Fíla, J. & Honys, D. (2016). Male gametophyte development and function in angiosperms: a general concept. *Plant Reproduction*, *29*(1-2), 31–51.

Inzé, D. & De Veylder, L. (2006). Cell cycle regulation in plant development. *Annual Review of Genetics*, *40*, 77–105.

Islam, S.M. & Tuteja, N. (2012). Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. *Plant Sciences*, *182*, 134–144.

Levin, D.E. (2005). Cell Wall Integrity Signaling in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, *69*(2), 262–291.

Leydon, A.R., Chaibang, A. & Johnson, M.A. (2014). Interactions between pollen tube and pistil control pollen tube identity and sperm release in the *Arabidopsis* female gametophyte. *Biochemical Society Transactions*, *42*(2), 340–345.

Li, H., Soriano, M., Cordewener, J., Muiño, JM., Riksen, T., Fukuoka, H., Angenent, G.C. & Boutilier, K. (2014). The histone deacetylase inhibitor trichostatin a promotes totipotency in the male gametophyte. *Plant Cell*, *26*, 195–209.

Li, K., Prada, J., Damineli, D.S.C., Liese, A., Romeis, T., Dandekar, T., Feijó, J.A., Hedrich, R. & Konrad, K.R. (2021). An optimized genetically encoded dual reporter for simultaneous ratio imaging of Ca^{2+} and H+ reveals new insights into ion signaling in plants. *New Phytologist*, *10*, 1111.

Li, W.L., Liu, Y. & Douglas, C.J. (2017). Role of glycosyltransferases in pollen wall primexine formation and exine patterning. *Plant Physiology*, *173*(1), 167–182.

Li, X., Li, L. & Yan, J. (2015). Dissecting meiotic recombination based on tetrad analysis by single-microspore sequencing in maize. *Nature Communications*, *24*(6), 6648.

Li, Y., Suen, D.F., Huang, C.Y., Kung, S.Y. & Huang, A.H. (2012). The maize tapetum employs diverse mechanisms to synthesize and store proteins and flavonoids and transfer them to the pollen surface. *Plant Physiology*, *158*(4), 1548–1561.

Li, Y., Li, L., Wang, Y., Wang, Y.C., Wang, N.N., Lu, R., Wu, Y.W. & Li, X.B. (2020). Pollen-specific protein PSP231 activates callose synthesis to govern male gametogenesis and pollen germination. *Plant Physiology*, *184*(2), 1024–1041.

Li, Y.Q., Faleri, C., Geitmann, A. Zhang, H.Q. & Cresi, M. (1995). Immunogold localization of arabinogalactan proteins, unesterified and esterified pectins in pollen grains and pollen tubes of *Nicotiana tabacum* L. *Protoplasma*, *189*, 26–36.

Liu, J., Zhang, Y., Qin, G., Tsuge, T., Sakaguchi, N., Luo, G., Sun, K., Shi, D., Aki, S., Zheng, N., Aoyama, T., Oka, A., Yang, W., Umeda, M., Xie, Q., Gu, H. & Qu, L.J. (2008). Targeted degradation of the cyclin-dependent kinase inhibitor ICK4/KRP6 by RING-type E3 ligases is essential for mitotic cell cycle progression during *Arabidopsis* gametogenesis. *Plant Cell*, *20*(6), 1538–1554.

Liu, L. & Wang, T. (2021). Male gametophyte development in flowering plants: A story of quarantine and sacrifice. *Journal of Plant Physiology*, *21*, 258–259.

Loubert-Hudon, A., Mazin, B.D., Chevalier, É. & Matton, D.P. (2020). The ScRALF3 secreted peptide is involved in sporophyte to gametophyte signalling and affects pollen mitosis I. *Plant Biology*, *22*(1), 13–20.

Luo, G., Gu, H., Liu, J. & Qu, L.J. (2012). Four closely-related RING-type E3 ligases, APD1-4, are involved in pollen mitosis II regulation in *Arabidopsis*. *Journal of Integrative Plant Biology*, *54*(10), 814–827.

Ma, H., Sundaresan, V. (2010). Development of flowering plant gametophytes. *Current Topics in Developmental Biology*, *91*, 379–412.

Mackenzie, G., Boa, A.N., Taboada, A.D., Atkin, S.L. & Sathyapalan, T. (2015). Sporopollenin, the least known yet toughest natural biopolymer. *Frontiers in Materials*, *2*, 66.

Maraschin, S.F., de Priester, W., Spaink, H.P. & Wang, M. (2005). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. *Journal of Experimental Botany*, *56*(417), 1711–1726.

Mariani, C., De Beuckeleer, M., Truettner, J., Leemans, J. & Goldberg, R.B. (1990). Induction of male sterility in plants by a chimeric ribonuclease gene. *Nature*, *347*, 737–741.

McCormick, S. (2004). Control of male gametophyte development. *Plant Cell*, *16*, 142–153.

Michard, E., Simon, A.A., Tavares, B., Wudick, M.M. & Feijó, J.A. (2017). Signaling with ions: the keystone for apical cell growth and morphogenesis in pollen tubes. *Plant Physiology*, *173*, 91–111.

Ning, L., Lin, Z., Gu, J., Gan, L., Li, Y., Wang, H., Miao, L., Zhang, L., Wang, B. & Li, M. (2018). The initial deficiency of protein processing and flavonoids biosynthesis were the main mechanisms for the male sterility induced by SX-1 in *Brassica napus*. *BMC Genomics*, *19*(1), 806.

Nowicka, A., Juzoń, K., Krzewska, M., Dziurka, M., Dubas, E., Kopeć, P., Zieliński, K. & Żur, I. (2019). Chemically-induced DNA de-methylation alters the effectiveness of microspore embryogenesis in triticale. *Plant Science*, *287*, 110189.

Pacini, E. & Dolferus, R. (2019). Pollen developmental arrest: Maintaining pollen fertility in a world with a changing climate. *Frontiers in Plant Science*, *10*, 679.

Pacini, E., Franchi, G.G. & Hesse, M. (1985). The tapetum: its form, function, and possible phylogeny in Embryophyta. *Plant Systematics and Evolution*, *149*, 155–185.

Parra-Vega, V., Corral-Martínez, P., Rivas-Sendra, A. & Seguí-Simarro, J.M. (2015). Formation and excretion of autophagic plastids (plastolysomes) in *Brassica napus* embryogenic microspores. *Frontiers in Plant Science*, *19*(6), 94.

Paxson-Sowders, D.M., Dodrill, C.H., Owen H.A., & Makaroff, C.H. (2001). DEX1, a novel plant protein, is required for exine pattern formation during pollen development in *Arabidopsis*. *Plant Physiology*, *127*(4), 1739–1749.

Pérez-Pérez, Y., El-Tantawy, A.A., Solís, M.T., Risueño, M.C. & Testillano, P.S. (2019 a). Stress-induced microspore embryogenesis requires endogenous auxin synthesis and polar transport in barley. *Frontiers in Plant Sciences*, *27*(10), 1200.

Pérez-Pérez, Y., Bárány, I., Berenguer, E., Carneros, E., Risueño, M.C. & Testillano, P.S. (2019 b). Modulation of autophagy and protease activities by small bioactive compounds to reduce cell death and improve stress-induced microspore embryogenesis initiation in rapeseed and barley. *Plant Signal & Behavior*, *14*(2), 1559577.

Rejón, J.D., Delalande, F., Schaeffer-Reiss, C., Alché J.D.D., Rodríguez-García, M.I, van Dorsselaer, A. & Castro, A.J. (2016). The pollen coat proteome: At the cutting edge of plant reproduction. *Proteomes*, *4*(1), 5.

Ringli, C. (2010). Monitoring the outside: cell wall-sensing mechanisms. *Plant Physiology*, *153*, 1445–1452.

Ruan, H., Li, J., Wang, T. & Ren, H. (2021). Secretory vesicles targeted to plasma membrane during pollen germination and tube growth. *Frontiers in Cell Developmental Biology*, *8*, 615447.

Schmidt, A., Wuest, S.E., Vijverberg, K., Baroux, C., Kleen, D. & Grossniklaus, U. (2011). Transcriptome analysis of the *Arabidopsis* megaspore mother cell uncovers the importance of RNA helicases for plant germline development. *PLoS Biology*, *9*(9), 1001155.

Scott, R.J., Spielman, M. & Dickinson, H.G. (2004). Stamen structure and function. *Plant Cell*, *16*, 46–60.

Seguí-Simarro, J.M. (2016). Doubled haploidy in model and recalcitrant species. *Frontiers in Plant Science*, *24*(6), 1175.

Sheoran, I.S., Pedersen, E.J., Ross, A.R. & Sawhney, V.K. (2009). Dynamics of protein expression during pollen germination in canola (*Brassica napus*). *Planta*, *230*(4), 779–793.

Shi, J., Cui, M., Yang, L., Kim, Y.J. & Zhang, D. (2015 a). Genetic and biochemical mechanisms of pollen wall development. *Trends in Plant Sciences*, *20*(11), 741–753.

Shi, X., Sun, X., Zhang, Z., Feng, D., Zhang, Q., Han, L., Wu, J. & Lu, T. (2015 b). GLUCAN SYNTHASE-LIKE 5 (GSL5) plays an essential role in male fertility by regulating callose metabolism during

microsporogenesis in rice. *Plant and Cell Physiology*, *56*(3), 497–509.

Solís, M.T., El-Tantawy, A.A., Cano, V., Risueño, M.C. & Testillano, P.S. (2015). 5-azacytidine promotes microspore embryogenesis initiation by decreasing global DNA methylation, but prevents subsequent embryo development in rapeseed and barley. *Frontiers in Plant Science*, *6*, 472.

Suzuki, T., Narciso, J.O., Zeng, W., van de Meene, A., Yasutomi, M., Takemura, S., Lampugnani, E.R., Doblin, M.S., Bacic, A. & Ishiguro, S. (2017). KNS4/UPEX1: A type II arabinogalactan β-(1,3)-galactosyltransferase required for pollen exine development. *Plant Physiology*, *173*(1), 183–205.

Testillano, P.S. (2019). Microspore embryogenesis: targeting the determinant factors of stress-induced cell reprogramming for crop improvement. *Journal of Experimental Botany*, *70*(11), 2965–2978.

Thompson, E.P., Wilkins, C., Demidchik, V., Davies, J.M. & Glover, B.J. (2010). An *Arabidopsis* flavonoid transporter is required for anther dehiscence and pollen development. *Journal of Experimental Botany*, *61*(2), 439–451.

Touraev, A., Ilham, A., Vicente, O. & Heberle-Bors, E. (1996 a). Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. *Plant Cell Reports*, *15*(8), 561-565.

Touraev, A., Indrianto, A., Wratschko, I., Vicente, O. & Heberle-Bors, E. (1996 b). Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. *Sexual Plant Reproduction*, *9*, 209-215.

Touraev, A., Pfosser, M., Vicente, O. and Heberle-Bors, E. (1996 c). Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. *Planta*, *200*, 144–152.

Tupy, J., Rihova, L. & Zarsky, V. (1991). Production of fertile tobacco pollen from microspores in suspension culture and its storage for *in situ* pollination. *Sexual Plant Reproduction*, *4*, 284–287.

Ünal, M., Vardar, F. & Ayturk, Z. (2013). Callose in plant sexual reproduction. InTech-*Current Progress in Biological Research*, 319–343.

Paxson-Sowders, D.M., Dodrill, C.H., Owen, H.A. & Makaroff, C.A. (2001). DEX1, a novel plant protein, is required for exine pattern formation during pollen development in Arabidopsis. *Plant Physiology*, *27*(4), 1739–1749.

van Eldik, G.J., Reijnen, W.H., Ruiter, R.K., van Herpen, M.M., Schrauwen, J.A. & Wullems, G.J. (1997). Regulation of flavonol biosynthesis during anther and pistil development, and during pollen tube growth in *Solanum tuberosum*. *Plant Journal*, *11*(1), 105–113.

Vaz Dias, F., Serrazina, S., Vitorino, M., Marchese, D., Heilmann, I., Godinho, M., Rodrigues, M. & Malhó, R. (2019). A role for diacylglycerol kinase 4 in signalling crosstalk during *Arabidopsis* pollen tube growth. *New Phytologist*, *222*(3), 434–446.

Wan, L., Zha, W., Cheng, X., Liu, C., Lv, L., Liu, C., Wang, Z., Du, B., Chen, R., Zhu, L. & He, G. (2011). A rice β-1,3-glucanase gene Osg1 is required for callose degradation in pollen development. *Planta*, *233*(2), 309–323.

Wang, B., Fang, R., Zhang, J., Han, J., Chen, F., He, F., Liu, Y.G. & Chen, L. (2020). Rice LecRK5 phosphorylates a UGPase to regulate callose biosynthesis during pollen development. *Journal of Experimental Botany*, *71*(14), 4033–4041.

Ylstra, B., Touraev, A., Moreno, R.M., Stöger, E., van Tunen, A.J., Vicente, O., Mol, J.N. & Heberle-Bors, E. (1992). Flavonols stimulate development, germination, and tube growth of tobacco pollen. *Plant Physiology*, *100*(2), 902–907.

Zhang, Y.C., He, R.R., Lian, J.P., Zhou, Y.F., Zhang, F., Li, Q.F., Yu, Y., Feng, Y.Z., Yang, Y.W., Lei, M.Q., He, H., Zhang, Z. & Chen, Y.Q. (2020). OsmiR528 regulates rice-pollen intine formation by targeting an uclacyanin to influence flavonoid metabolism. *Proceedings of the Natural Academy of Sciences*, *117*(1), 727–732.

Zarsky, V., Garrido, D., Rihova, L., Tupy, J., Vicente, O. & Heberle-Bors, E. (1992). Derepression of the cell cycle by starvation is involved in induction of tobacco pollen embryogenesis. *Sexual Plant Reproduction*, *5*, 189–194.

Zur, I., Dubas, E., Golemiec, E., Szechyńska-Hebda, M., Gołębiowska, G. & Wedzony, M. (2009). Stress-related variation in antioxidative enzymes activity and cell metabolism efficiency associated with embryogenesis induction in isolated microspore culture of triticale (× *Triticosecale* Wittm.). *Plant Cell Reports*, *28*, 1279–1287.