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REVIEW

Microspore embryogenesis in *Brassica*: calcium signaling, epigenetic modification, and programmed cell death

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Abstract

Main conclusion **Stress induction followed by excessive calcium influx causes multiple changes in microspores resulting in chromatin remodeling, epigenetic modifications, and removal of unwanted gametophytic components via autophagy, switching microspores towards ME.**

In *Brassica*, isolated microspores that are placed under specific external stresses can switch their default developmental pathway towards an embryogenic state. Microspore embryogenesis is a unique system that speeds up breeding programs and, in the context of developmental biology, provides an excellent tool for embryogenesis to be investigated in greater detail. The last few years have provided ample evidence that has allowed *Brassica* researchers to markedly increase their understanding of the molecular and sub-cellular changes underlying this process. We review recent advances in this field, focusing mainly on the perception to inductive stresses, signal transduction, molecular and structural alterations, and the involvement of programmed cell death at the onset of embryogenic induction.

Keywords Methylation · Microspore-derived embryo · Programmed cell death · Suspensor

Introduction

In plants, microsporogenesis, the formation of haploid microspores from pollen mother cells inside microsporangia, followed by microgametogenesis, the development of microspores after tetrad release, are two well-studied phenomena leading to the generation of functional pollen (Lu et al. 2014; Sharma et al. 2015). Upon specific stress treatment(s), the default developmental pathway of a pollen grain precursor, the microspore, can be switched and reprogrammed towards a new embryogenic state followed by sporophytic divisions giving rise to globular, heart, torpedo, and finally a haploid or doubled haploid (DH) microspore-derived embryo (MDE) (Fig. 1), a process that is referred to as "microspore embryogenesis" (ME) (Touraev et al. 1997). ME is an excellent model system for accelerating breeding programs, quantitative trait loci (QTL) mapping, studying the molecular mechanisms controlling ME, and in reverse breeding (Shariatpanahi and Ahmadi 2016). ME is highly genotype dependent and shows wide efficiency among crop species, with the *Brassica* genus, especially *B. napus*, very responsive to this process. *B. napus* 'Topas' is the cultivar of choice and often serves as a model to optimize isolated microspore culture since more than 10% of cultured microspores form MDEs (Ferrie 2003; Malik et al. 2007), whereas in *B. rapa* and *B. oleracea* less than 1% of microspores convert to MDEs (Lee et al. 2014; Zeng et al. 2015a; Bhatia et al. 2018). Over the past few decades, ME has been extensively studied in *Brassica* species and has now become one of the best models for studying androgenesis and the mechanisms underlying this process. Besides genotype, the efficiency of ME is also greatly affected by the growth condition of donor plants, the developmental stage of harvested microspores, the composition of the in vitro culture medium, and the type of external stressor that is applied (Ferrie and

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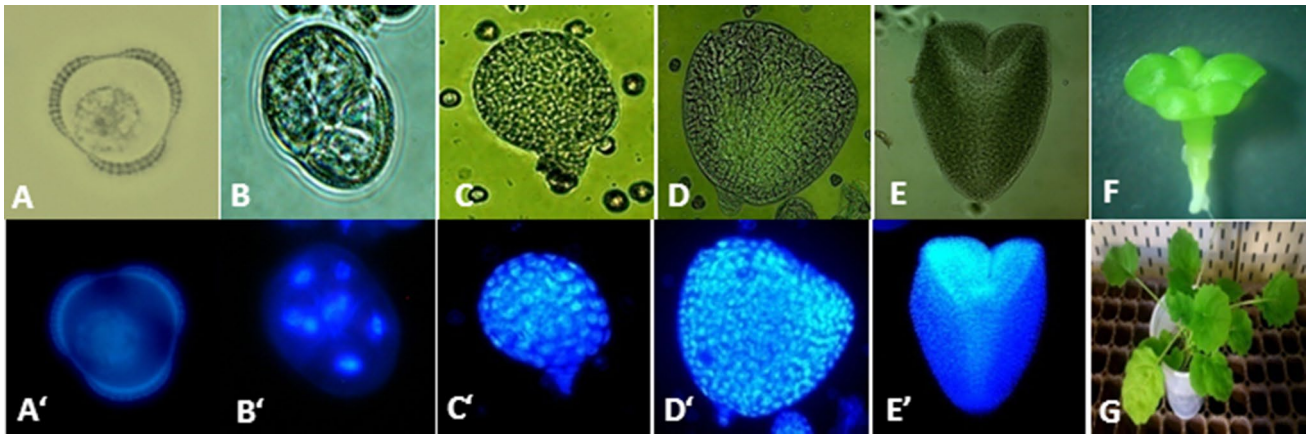

Fig. 1 Microspore embryogenesis (ME) in *Brassica napus* L. **a** Freshly isolated microspore at mid- to late-uninucleate stage; **b** derived pro-embryo; **c–e** globular-, heart-, and torpedo-shaped pro-embryo, respectively; **f** microspore-derived embryo (MDE); **g** regenerated plant. **a'–e'** DAPI staining of corresponding MDE developmental stages

Caswell 2011; Ahmadi et al. 2012, 2014a, b; Hoseini et al. 2014; Ahmadi and Shariatpanahi 2015).

In *Brassica*, pre-treatment of donor plants with low temperature (5–15 °C) prior to flowering until harvesting of floral buds, or exposure of harvested buds to cold (4 °C) for few days (Table 1), is a prerequisite for the androgenic switch and can increase the efficiency of ME (Gu et al. 2004, 2014a; Dubas et al. 2013; Bhatia et al. 2017). Dubas et al. (2013) showed that the application of a heat shock (32 °C) could not induce embryogenesis in microspores harvested from donor plants of *B. napus* growing under optimal physiological conditions (18 °C), but could induce ME only in microspores that were harvested from donor plants grown under lower temperature (10 °C). However, the precise mechanism(s) underlying the combined effect of cold pre-treatment of donor plants or floral buds and heat-stressed microspores on the efficiency of ME remains unclear. High plasma membrane fluidity and increased levels of abscisic acid (ABA), a stress-related hormone, have been reported in cultured microspores harvested from cold-pretreated donor plants, suggesting a relationship between cold treatment, the physical property of the microspore membrane, and endogenous ABA concentration (Dubas et al. 2013; rev. Żur et al. 2015). ABA and other stress hormones such as salicylic acid and jasmonic acid appear to play a role in ME-inducing signal transduction (Ahmadi et al. 2014a; rev. Żur et al. 2015) but the exact mechanism is still not fully understood.

The efficiency of ME also depends on the developmental stage of cultured microspores at the time of harvest. In *Brassica*, microspores at the mid- to late-uninucleate stage and even early bicellular pollen microgametophytes can switch efficiently to a sporophytic pathway (Table 1), but the exact window of competence is species- and genotype-specific (Ferrie and Caswell 2011; Seguí-Simarro et al. 2011; Soriano et al. 2013; Rivas-Sendra et al. 2017). The identification and isolation of this developmental stage is essential to setting up a successful ME system because this is when microspores are most sensitive to inducing treatments.

The application of an external stress is also necessary for the initiation of ME, but this depends on the plant species, various stresses, including heat and cold shock, osmotic stress, starvation, depolymerizing agents, heavy metals and growth regulators, alone or in combination, which can be used to induce androgenesis (Shariatpanahi et al. 2006; Islam and Tuteja 2012). In *Brassica*, ME is usually efficiently induced after exposing in vitro-cultured microspores to a mild heat shock (30–33 °C) for 1 day up to 2 weeks (Table 1). Besides inducing ME, the intensity and duration of the heat shock also determines the mode of MDE generation, i.e., whether it develops a suspensor or not (Soriano et al. 2013).

Suspensor-bearing vs. suspensor-less MDEs

Not all cultured microspores respond to inductive stresses, and a few days after initiation, they typically contain four types of co-existing structures: (a) small tri-lobed cells, which are arrested, developing either gametophytically or sporophytically and finally die; (b) oblong-shaped cells with a dense cytoplasm filled with starch that follow the original gametophytic route, dividing asymmetrically, and producing the generative and vegetative nuclei typical of pollen grains; (c) non-embryogenic structures, microspores that undergo a few divisions, with enlarged cells of variable sizes that finally become arrested, not showing any sign of change or progression at later stages; (d) embryogenic structures that are often formed by a series of randomly oriented divisions within the surrounding exine wall (pro-embryos) that finally emerge and give rise to globular, heart, torpedo, and cotyledonary MDEs (Fig. 2) (Corral-Martínez et al. 2013;

Table 1 The condition of donor plant growth, flower bud pre-treatment, developmental stage of microspores, culture medium, and type of inducing stressor applied for induction of microspore embryogenesis in different *Brassica* species

Species	Donor plant growth condition	Flower bud pre-treatment	Developmental stage of microspores	Culture medium	Inducing stress	References
B. napus	10/5 °C day/night temp	–	Late uninucleate–early binucleate	NLN-13	32 °C for 3 days	Bhowmik et al. (2011)
	15/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	Continuously 32 °C or 18 °C	Prem et al. (2012) and El-Tantawy et al. (2013)
	15/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	30 °C for 14 days	Abdollahi et al. (2012) and Mohammadi et al. (2012)
	15/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	30 °C for 12–14 days	Ahmadi et al. (2014a, b), Ahmadi and Shariatpanahi (2015) and Ahmadi et al. (2016)
	15/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	30 °C for 18 days	Hoseini et al. (2014)
	16/10 °C day/night temp	–	Late uninucleate–early binucleate	NLN-13	32.5 °C for 1 day	Leroux et al. (2016)
	10/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	32 °C for 1 day	Dubas et al. (2013)
B. rapa	12/10 °C day/night temp	–	Late uninucleate–early binucleate	NLN-10	32.5 °C for 1 day	Kabir et al. (2013)
	15/15 °C day/night temp	–	Late uninucleate–early binucleate	NLN-13	32 °C for 2 days	Shumilina et al. (2015)
	25/15 °C day/night temp	–	Mid to late uninucleate	NLN-13	33 °C for 1–2 days	Zhang et al. (2011), (2012), (2016)
	Greenhouse	4 °C for 10–20 days	Late uninucleate–early binucleate	1/2NLN-10	32.5 °C for 1 day	Takahashi et al. (2012)
B. oleracea	25/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	33 °C for 1 day	Wang et al. (2011)
	10/10 °C day/night temp	–	Mid to late uninucleate	NLN-13	30.5 °C for 2 days	Winarto and Teixeira da Silva (2011)
	23/12 °C day/night temp	4 °C for 1–2 days	Late uninucleate–early binucleate	1/2NLN-13	32.5 °C for 1 day	Yuan et al. (2011)
	23/12 °C day/night temp		Late uninucleate–early binucleate	NLN-13	32.5 °C for 1 day	Yuan et al. (2012)
	Greenhouse	4 °C for 1–4 days	Mid to late uninucleate	NLN-13	32–33 °C for 1 day	Gu et al. (2014a)
	25/14 °C day/night temp	4 °C for 1–3 days	Late uninucleate	NLN-13	32.5 °C for 1 day	Zeng et al. (2015b)
	Field	–	Late uninucleate–early binucleate	NLN-13	30–32.5 °C for 1–2 days	Bhatia et al. (2018)
	Greenhouse	–	Late uninucleate	NLN-13	30 °C for 2 days	Pilih et al. (2018)
B. nigra	Field	–	Late uninucleate	NLN-13	32 °C for 1 day	Gu et al. (2014b)

Li et al. 2014; Soriano et al. 2013, 2014; Dubas et al. 2014; Parra-Vega et al. 2015a; Solís et al. 2015). The generation of suspensor-less MDEs is the most commonly observed pathway of ME in *Brassica*. Suspensor-bearing MDEs, on the other hand, no longer initially develop by random cell division but rather follow a highly regular cell division pattern typical for *B. napus* zygotic embryos (Soriano et al. 2013, 2014; Tang et al. 2013). Suspensor-bearing MDEs are basically generated via two distinct patterns: in the first pattern, both daughter cells undergo transverse divisions after the first asymmetric division following which the apical cell undergoes a longitudinal division and finally develops into the embryo proper, while the basal cell continues to undergo transverse divisions to form the suspensor. In the second pattern, after the first asymmetric division, one daughter cell generates a long uniseriate cell that emerges through a pore

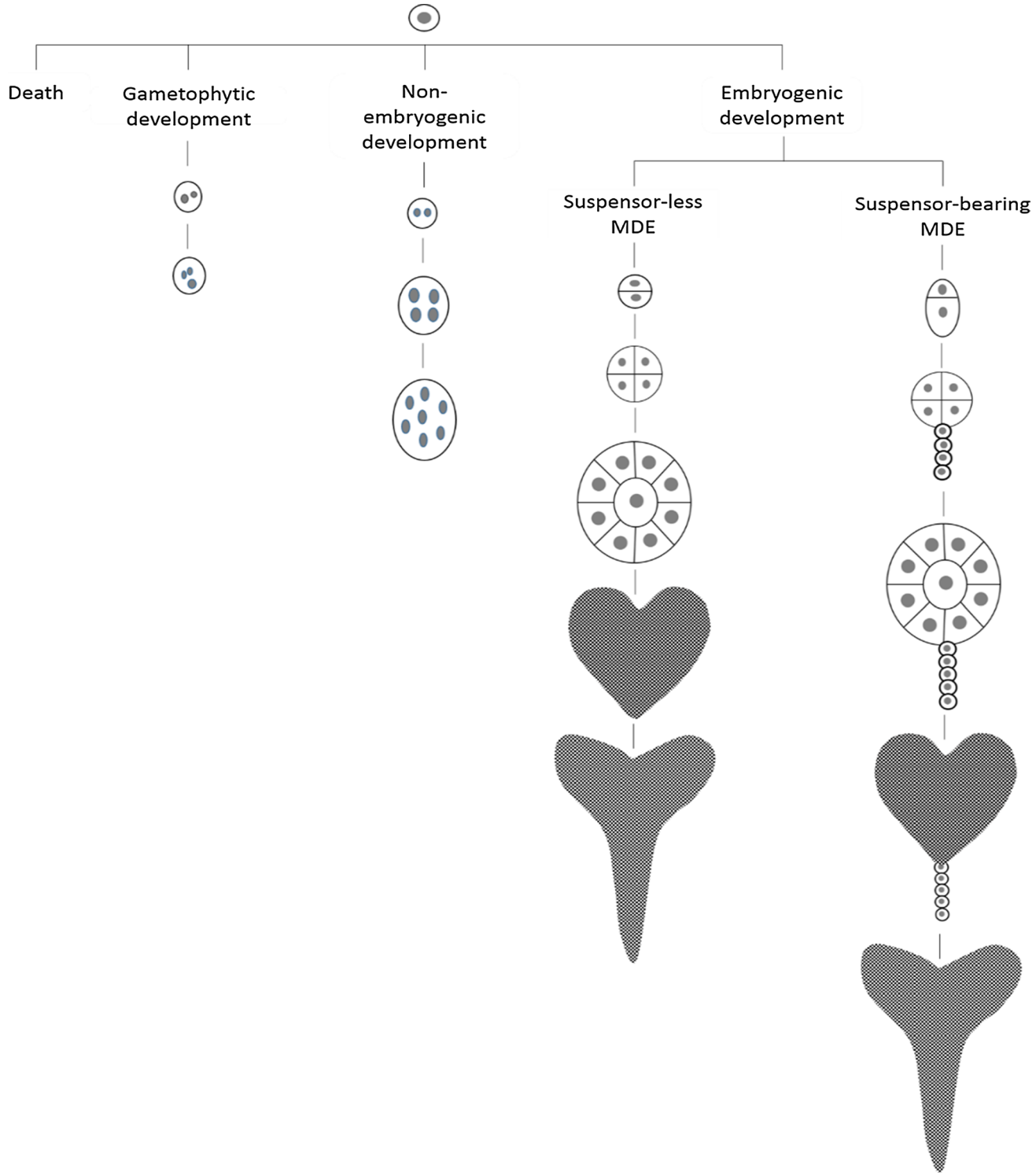

Fig. 2 Different fates of in vitro-cultured *Brassica* microspores. The majority of microspores die after exposing to heat as inducing treatment. Some others still continue their original developmental pathway forming mature pollen grains in culture medium. Some microspores enter non-embryogenic development followed by few sporophytic divisions, but never become MDEs. A small fraction of cultured microspores switch to embryogenesis giving rise to MDEs either with or without a suspensor

in the exine wall by transverse divisions giving rise to the suspensor while the embryo proper develops from the distal cell of the filament, first dividing longitudinally and then in an ordered pattern which develops into globular, heart, and torpedo MDEs that are similar to zygotic embryos. The suspensor has a varied form, appearing either as a long filament or redundant cells, visualized as an irregular protuberance at the radical pole of the MDEs (Tang et al. 2013).

The efficiency of suspensor formation in microspore culture is mostly determined by genotype, as well as the type and intensity of the inducing stressor (Soriano et al. 2013; Tang et al. 2013). MDEs with a suspensor are efficiently generated in the model *B. napus* cultivar 'Topas' by exposing cultured microspores either to a short or mild heat stress (12–72 h at 32 °C) (Dubas et al. 2014; Soriano et al. 2014) or by continuously maintaining them at a low temperature (18 °C) (Prem et al. 2012), but exposure to higher or lower temperatures or prolonged periods of incubation can erase polarity cues and result in symmetric division of the microspore and the formation of randomly divided structures (Prem et al. 2012; Dubas et al. 2014; Soriano et al. 2014). The suspensor plays a crucial role in proper embryo patterning through auxin biosynthesis (Prem et al. 2012; Dubas et al. 2014; Rodríguez-Sanz et al. 2015). Besides auxin production, suspensor cells also exhibit embryogenic potential so that the embryo proper, in some cases, forms and develops in the middle of the filament suspensor (Supena et al. 2008; Liu et al. 2015) and gives rise to genetically identical twin MDEs joined either at the base of the hypocotyl or along the length of the hypocotyl axis (Cousin and Nelson 2009). Suspensor cells cease division and often degenerate after the globular or heart stage (Soriano et al. 2013; Tang et al. 2013) showing that the suspensor supports early cellular patterning in the embryo proper and does not participate in later development of embryos (Liu et al. 2015).

The last decade has provided ample evidence and increased our understanding of the molecular and sub-cellular changes at the initial stages of the androgenic switch in *Brassica*. This review highlights the past and current status of research on calcium signaling, chromatin remodeling and epigenetic modification, and also programmed cell death (PCD) following stress application in this process.

Calcium signaling

To switch to androgenesis, in vitro-cultured *Brassica* microspores are often subjected to a heat stress treatment. Heat shock tends to fluidize plasma membranes (Pauls et al. 2006; Dubas et al. 2013; Parra-Vega et al. 2015a), making ions leaky and thus favoring influxes of calcium ions and rapid, transient, and proportional elevations of intracellular calcium in embryogenic microspores, i.e., microspores that have acquired embryogenic competence but have not yet become MDEs (Parra-Vega et al. 2015a; Rivas-Sendra et al. 2017). It has also been hypothesized that calcium could be released into the cytoplasm from sub-cellular organelles such as the endoplasmic reticulum, vacuoles or mitochondria with the aid of inositol triphosphate (IP_3) and diacylglycerol (DAG) that are produced by the action of phospholipase C (PLC) on phosphatidylinositol-4,5-bisphosphate (PIP_2) in the cell wall membrane (Pauls et al. 2006). The increased level of intracellular calcium is then decoded and transmitted by a toolkit of calcium-binding proteins such as calmodulins (CaMs), calmodulin-like proteins (CaMLs), calcineurin B-like proteins (CBLs), calcium-dependent protein kinases (CPKs), and mitogen-activated protein kinases (MAPKs) that relay signals into the nucleus and thereby alter the default developmental pathway of microspores towards embryogenesis (Seguí-Simarro et al. 2005; Pauls et al. 2006; Tsuwamoto and Takahata 2008). Even though the importance and the enhancing effect of exogenously applied calcium in culture medium on the efficiency of MDE production has been discussed in different plant species (Reynolds 2000; Tian et al. 2004; Broughton 2011; Leroux et al. 2016), its precise role throughout ME is still ambiguous. Embryogenesis-committed microspores exhibit an altered level of intracellular calcium and a different pattern of distribution: 1 day after subjecting cultures to heat stress (32 °C), intracellular calcium level increased dramatically (~ 2.5-fold increase in nuclear–cytosolic calcium and ~ 19-fold increase in vacuolar calcium level) in competent microspores, the majority of which were internalized in vacuoles (Rivas-Sendra et al. 2017). Vacuolar storage of excess calcium in embryogenic microspores is most likely a mechanism to maintain calcium homeostasis under control and, therefore, to avoid toxicity or death (Rivas-Sendra et al. 2017). However, as derived pro-embryos develop further, cytosolic and vacuolar calcium levels continue to decline (Rivas-Sendra et al. 2017). Unlike embryogenic microspores, dividing cells that follow a callus-like pathway exhibit a distribution pattern mostly like gametophytically developing microspores, always being cytosolic and not vacuolar (Rivas-Sendra et al. 2017). Increased intracellular calcium level also affects cell wall architecture, arrangement and composition, including the development of a sub-intinal layer—a callose rich but cellulose-deficient layer beneath the intine and the plasma membrane with alternating thick and thin domains—in the microspores as they enter embryogenesis (Parra-Vega et al. 2015a; Rivas-Sendra et al. 2017). The sub-intinal layer dismantles when calcium level declines in multicellular structures and MDEs with a clearly established embryogenic pattern in which callose is progressively substituted by cellulose and can serve as a novel primary sign of the commitment to embryogenesis (Parra-Vega et al. 2015a; Rivas-Sendra et al. 2017). Chemical modulators of various steps in the calcium

signaling pathway have long been considered as precious tools to study the roles of this ion. Upon addition of 1 mM caffeine to NLN-13 culture medium (Lichter 1982), which reduces the intracellular calcium levels by altering membrane permeability, thinner callose-containing cell walls are observed in daughter cells than in control cells. In turn, the use of 100 µM of benzyl alcohol, a membrane fluidizer that induces major but transient elevations of cytosolic calcium, produces even more callose in the sub-intinal layer, most likely due to an increase in calcium levels, suggesting a possible relationship between the abundance of intracellular calcium and the amount of deposited callose (Parra-Vega et al. 2015a). Increased intracellular calcium acts by enhancing the activity of the callose synthase complex and thereby regulating callose deposition in plant cells (Nedukha 2015). Excessive callose deposition on the cell wall may also impair the cytoskeleton and generate a structural context necessary to facilitate nuclear fusion and, therefore, genome duplication, a necessary step widely needed to produce DH embryos (Parra-Vega et al. 2015a). These lines of evidence indicate that intracellular calcium level increases rapidly in embryogenic committed microspores leading to massive deposition of callose by activating calcium-dependent callose synthase as a direct consequence of increased permeability of plasma membrane following exposure to heat shock.

Chromatin remodeling and epigenetic modifications

Chromatin remodeling, the dynamic modification of chromatin architecture that allows access of condensed genomic DNA to regulatory elements, occurs as a consequence of all plant developmental switches, including ME (Seguí-Simarro et al. 2011; Rodríguez-Sanz et al. 2014a). In eukaryotic cells, DNA is packaged by forming fundamental repeating units, each composed of an octamer of core histones (H2A, H2B, H3 and H4), to generate chromatin which is further compacted to form the chromosome (Temel et al. 2015). A direct relationship between nuclear architecture and the level of cellular activity typically coexists so that in compacted structures, DNA transcription and gene expression are inactivated. Unlike gametophytically developing microspores which contain a highly condensed chromatin pattern, microspores committed to embryogenesis exhibit a slightly larger nucleus and decondensed chromatin patterns, allowing for access of the interchromatin domain to various transcription factors during proliferation and differentiation events (Seguí-Simarro et al. 2011; Solís et al. 2012; Rodríguez-Sanz et al. 2014a). This flexibility of the genome and accessibility of genes to the transcriptional machinery are accompanied by epigenetic modifications, heritable changes in gene expression not encoded by the DNA sequence, such as DNA methylation and histone modifications (Solís et al. 2012, 2015; Testillano et al. 2013; Temel et al. 2015). Quantification of global DNA methylation in *B. napus* by high-performance capillary electrophoresis and the 5-methyldeoxycytidine immunolocalization assay indicated a low level of DNA methylation in pro-embryos not significantly different from vacuolated microspores (Table 2) (4.33 vs. 3.02%, respectively) but a steady and significant increase after the transition of derived pro-embryos to globular (6.67%) and cotyledonary MDEs (12.27%) (Solís et al. 2012). Besides its dynamic nature, DNA methylation also exhibits a differential distribution pattern throughout the development of the MDE (Solís et al. 2012). Vacuolated microspores and pro-embryos show a similar and punctuated pattern of distribution at the periphery of the nucleus while in globular, torpedo and cotyledonary MDEs, methylation exists at the highest level in differentiating cells located in the outer layers forming the epidermis than in proliferating cells in the inner part of MDEs indicating that cell differentiation is accompanied by

Table 2 Epigenetic modifications throughout microspore embryogenesis in *Brassica* spp.

MDE stage	Globular				Heart	Cotyledonary	References
Epigenetic modifications							
DNA methylation	+	−	+	++	+++	+++	Solís et al. (2012) and Rodríguez-Sanz et al. (2014a)
H3K9 methylation	+	++	++	+++	+++	+++	Rodríguez-Sanz et al. (2014b) and Berenguer et al. (2017)
H3/H4 acetylation	+	−	−	+	+	+	Rodríguez-Sanz et al. (2014b)

Vacuolated microspores undergo a plethora of changes giving rise to star-like structure, then undergo sporophytic divisions and progress into globular, heart, and cotyledonary MDE

−, +, ++, +++ not detected, weakly, moderately, and highly detected, respectively

a rapid increase in global DNA methylation levels, as found in other systems (Costa and Shaw 2006; Solís et al. 2012).

DNA methylation could be inhibited in living cells by inhibiting DNA methyl transferase activity using epigenetic drugs such as 5-azacytidine (AzaC) and decitabine (Gnyszka et al. 2013), providing an important tool for in vivo studies of DNA methylation. Inhibiting DNA demethylation at initial stages promotes microspore reprogramming, the acquisition of totipotency, and the initiation of embryogenesis (Solís et al. 2015). Subjecting isolated microspores of *B. napus* to 2.5 µM AzaC for 4 days decreased global DNA methylation by half and reduced the number of dead cells but concomitantly increased the percentage of microspore-derived pro-embryos produced compared with control cultures (20% vs. 15%, respectively) while a longer duration of treatment (initial 30 days) caused a drastic decrease in the total number of MDEs produced (Solís et al. 2015). This indicates that reprogramming microspores to the sporophytic route is associated with a decrease in global DNA methylation (hypo-methylation) at initial stages but an increase in global DNA methylation (hyper-methylation) upon cell differentiation and progression of embryo differentiation (Solís et al. 2012, 2015; El-Tantawy et al. 2014; Rodríguez-Sanz et al. 2014a; Li et al. 2016).

Histone modifications, including methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation of histone proteins, are also among the major epigenetic events regulating gene expression during plant development and in response to environmental changes (Bannister and Kouzarides 2011). Histone H3 modification of lysine at position 9 (H3K9) is one of the most extensively studied histone modifications, with major functions in heterochromatin maintenance and genome-wide transcriptional silencing (Liu et al. 2010; Rodríguez-Sanz et al. 2014b; Berenguer et al. 2017). H3K9, once methylated or deacetylated, can easily silence transcriptionally active genes and can turn them on when acetylated during plant development and differentiation (reviewed by Boycheva et al. 2014; Brusslan et al. 2015). Induction of ME up to the formation of pro-embryos involves low levels of H3K9 methylation, but more developmentally advanced embryos, such as globular and torpedo MDEs, exhibit a marked increase, more than twofold the level found in initial stages (Table 2) (Rodríguez-Sanz et al. 2014b; Berenguer et al. 2017). Inhibiting H3K9 methylation with "BIX-01294", a diazepin-quinazolin-amine derivative and specific inhibitor of histone methylation, for 4 days, increased the proportion of pro-embryos produced (~ twofold increase) but the progression of ME ceased completely after prolonged treatment (30 days), showing that H3K9 methylation is not required for the initiation of ME but is crucial for the differentiation and development of MDEs (Berenguer et al. 2017). H3K9 methylation also exhibits a punctuate pattern of distribution so that in cotyledonary MDEs, higher H3K9 methylation exists in the nuclei of differentiating peripheral cells than in the nuclei of cells in other regions of proliferation, suggesting that cell differentiation is accompanied by increased H3K9 methylation (Rodríguez-Sanz et al. 2014b).

Histone acetylation, a process by which the lysine residues within the N-terminal tail protrude from the histone core (mainly H3 and H4 proteins) of the nucleosome, is also involved throughout ME induction and progression (Li et al. 2014; Rodríguez-Sanz et al. 2014b). Vacuolated microspores involve a high level of H3/H4 acetylation followed by a marked decrease after ME induction in multicellular structures and then increases again in advanced stages (Table 2) (Rodríguez-Sanz et al. 2014b). Increasing histone acetylation using histone deacetylase inhibitors such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (NaB) at the onset of ME have increased the rate of sporophytically diving cells and total number of regenerated MDEs in different plant species (Liu Mohammadi et al. 2012; Zhang et al. 2016; Jiang et al. 2017). In *B. napus*, only 6% of microspores divided sporophytically 2 days after exposure to heat stress (33 °C) but its combination with TSA substantially increased sporophytic division by as much as 80% (Li et al. 2014). The enhancing effects of histone deacetylase inhibitors on ME has also been reported in *B. rapa* whereby TSA (0.05 µM), SAHA (0.05–0.1 µM) and NaB (2.0 µM) increased ME by 2.48-, 4.35-, and 3.09-fold, respectively, when exogenously applied to the culture medium (Zhang et al. 2016). The addition of histone deacetylase inhibitors per se in culture medium was also sufficient enough to induce ME in the absence of heat stress by promoting histone acetylation (Li et al. 2014). Interestingly, treatment with histone deacetylase inhibitors and heat stress both mediate similar developmental changes in cultured microspores suggesting that heat stress used to induce ME impinges on pathways that are controlled by histone deacetylase proteins (Li et al. 2014).

Taken together, global DNA hypo-methylation and increasing histone acetylation are both required for microspore reprogramming, the acquisition of totipotency and the initiation of embryogenesis leading to an increased hyper-proliferation by increasing chromatin decondensation and facilitating the access of transcription factors to the chromatin fiber, especially those driving changes in the developmental program (Berenguer et al. 2017). Advanced stages of ME, namely globular, torpedo and cotyledonary MDE formation, on the other hand, are mainly accompanied by cell differentiation and, therefore, silencing of certain genes needed for rapid proliferation but expression of specific cell-type programs. Gene silencing associated with heterochromatization at later stages of ME (Solís et al. 2012; Rodríguez-Sanz et al. 2014b) may be controlled by DNA

methylation via chromatin modification (Berenguer et al. 2017).

Programmed cell death and autophagy

In plants, PCD, the death of a cell in any form mediated by an intracellular program, plays a critical role in the control of diverse developmental processes such as embryogenesis, seed development, seed germination, xylogenesis, pollen maturation, and leaf senescence (Kurusu and Kuchitsu 2017). Also, under stress conditions, targeted sacrifice of unwanted cells is crucial for maintaining total cell number and homeostasis. This process of targeted elimination or removal of selective cells or organelles by physiologically controlled death is collectively referred to as PCD (Ambastha et al. 2015). In multicellular organisms, autophagy and apoptosis are recognized as the two principal means by which PCD occur. Autophagy represents a process during which cytoplasmic content undergoes the degradation by vacuoles or plastids and is a mechanism commonly utilized by eukaryotic cells to recycle dysfunctional components and maintain cellular homeostasis (Mukhopadhyay et al. 2014). In plants, at least two defined types of autophagy, namely macro- and micro-autophagy, typically coexist. Macro-autophagy is used primarily to eradicate useless or damaged cell organelles by delivering cytoplasmic cargo to the vacuoles through the intermediary of double membrane-bound vesicles, referred to as autophagosomes (Lv et al. 2014; Wang et al. 2018). In micro-autophagy, by contrast, cytosolic components are directly taken up by the vacuole itself (Doorn and Papini 2013). Concreted evidence indicates that epigenetic modifications contribute to the control of autophagy and cell fate decision, being an integral part of the autophagic process (reviewed by Baek and Kim 2017).

During the last few years, many efforts have been made to obtain a detailed view of sub-cellular events taking place in the establishment of ME. Upon exposure to inducing stresses, embryogenic microspores undergo a plethora of changes such as nucleus repositioning to the cell center, vacuole fragmentation, a reduction in the number of ribosomes and plastids, starch granules, and lipid bodies, and the presence of organelle-free regions leading to cytoplasm clearance and the formation of star-like structures about 2–3 days following initial culture (reviewed by Soriano et al. 2013). Massive processes of autophagy are responsible for cytoplasm clearance exclusively in embryogenic microspores, but not in other non-induced microspores and also in non-embryogenic structures (Hosp et al. 2007; Rodríguez-Serrano et al. 2012; Corral-Martínez et al. 2013; Parra-Vega et al. 2015b; Bárány et al. 2018). Instantly stabilizing microspores by combining high-pressure freezing (HPF) and freeze substitution (FS), which fixes microspores in milliseconds without changing their original structure (Corral-Martínez et al. 2013; Parra-Vega et al. 2015b), as observed by 2-D TEM, indicated the presence of some membranous elements in embryogenesis-committed microspores, engulfing cytoplasmic regions containing different types of organelles, equivalent to those described as autophagosomes (Corral-Martínez et al. 2013). Two types of plastids with varying sizes and shapes were also observed in embryogenic microspores, both invaginating cytoplasmic regions (Parra-Vega et al. 2015b). The first type is the so-called "C- or dumbbell-shaped" plastids that trap portions of the cytoplasm, wrap around and eventually fuse their opposite ends to engulf it, and simply contain ribosomes and vesicles but are free of other organelles. The second type of plastids are those that are relatively round in shape and engulf a larger portion of the cytoplasm, some of which become deformed after engulfment and form atypical plastid profiles. Even though some of these cytoplasm-containing plastids show no structural evidence of digestion, many of them enter an autolytic process via acid phosphatase activity, leading to entire degradation of the plastid, showing that atypical plastids can act as plastolysomes and develop internal autophagic compartments that eventually lead to digestion of the entire plastid (Parra-Vega et al. 2015b). However, plastidial changes are a transient phenomenon occurring at initial stages of the androgenic switch since plastids in more advanced microspore-derived structures such as globular, heart, torpedo, and cotyledonary stages are very similar to those found in pollen grains with no engulfed cytoplasmic regions (Parra-Vega et al. 2015b). Amyloplasts in advanced microspores and pollen grains are unable to transform into plastolysomes, and would not be able to enter into the cytoplasmic cleaning process as extensively as those having proplastids, which may explain why more advanced and differentiated microspores cannot enter embryogenesis (Parra-Vega et al. 2015b). In addition to membranous elements and atypical plastids, some multi-lamellar bodies also exist in induced microspores and multicellular embryogenic structures that either independently digest or excrete different types of organelles and dense cytoplasmic materials to the apoplastic space by fusing their outer membrane with the plasma membrane (Corral-Martínez et al. 2013; Parra-Vega et al. 2015b). Based on these lines of evidence, both micro- and macro-autophagy co-exist in embryogenic microspores; however, according to the relative abundance of their profiles, macro-autophagy is the preferred pathway for cytoplasm cleaning in induced microspores (Corral-Martínez et al. 2013). In common plant autophagy, autophagosomes fuse with lytic vacuoles, which serve as the major site for the degradation and recycling of autophagosomal products, whereas useless cell materials of embryogenic microspores are usually secreted outside the cytoplasm, between the plasma membrane and the

cell wall (Corral-Martínez et al. 2013). The most reasonable explanation for these phenomena is the prevention of excessive growth of the vacuolar system followed by cell collapse and also to ensure proper embryogenic development since excessive vacuolation disrupts cell polarity needed for proper division and further development (Corral-Martínez et al. 2013). The degradation or removal of gametophytic cellular components or organelles and proteins is a prerequisite for switching the default original developmental fate of in vitro-cultured microspores towards an embryogenic state and autophagy would act as a cleaning mechanism for massive removal of useless cytoplasmic materials necessary to adapt the embryogenic microspore to its new developmental niche and enable the fittest microspores to de-differentiate and develop as embryos (Corral-Martínez et al. 2013; Parra-Vega et al. 2015b).

Conclusions and future perspectives

Under normal in vivo conditions, microspores develop gametophytically into pollen but, upon specific stress treatments, mainly heat stress in *Brassica*, cultured competent microspores can shift towards an embryogenic state giving rise to MDEs. Heat stress followed by excessive calcium influx causes multiple changes in microspores resulting in chromatin remodeling, epigenetic modifications, and the removal of unwanted gametophytic sub-cellular components via autophagy, finally switching microspores towards ME. Even though an overall scheme of ME has been drawn over the last decade, there is still a large gap in the step-wise events that occur in decoding calcium signals as secondary messengers, transducing this information to the nuclei and thereby altering gene expression. Moreover, although epigenetic modifications are involved in the form of DNA methylation, histone methylation and acetylation are well documented throughout ME, the possible regulatory role of other forms of DNA modifications such as phosphorylation and ubiquitination, as well as RNA-mediated epigenetic regulations, require further investigation. A more recent study reported the implication of *ATG* genes in the activation of autophagy at the onset of ME in *Hordeum vulgare* (Bárány et al. 2018). Even though the involvement of the target of the rapamycin (TOR) signal leading to the activation of *ATG* genes involved in autophagosome formation has been well studied in plants that encounter stress, the possible roles of TOR signaling throughout ME have not been investigated yet. Furthermore, the possible role of chaperone-mediated autophagy as a distinct autophagic pathway has yet to be studied.

Author contribution statement All authors whose names appear on the submission have contributed sufficiently to the scientific work and, therefore, share collective responsibility and accountability for the results.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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