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4 **Microspore embryogenesis: targeting the determinant factors of stress-**
5 **induced cell reprogramming for crop improvement**
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8 Pilar S. Testillano
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10 Pollen Biotechnology of Crop Plants group. Biological Research Center, CIB-CSIC.
11 Ramiro de Maeztu 9, 28040 Madrid. Spain

12 E-mail: testillano@cib.csic.es

13 Phone: +34-918373112 (Ext: 4366)
14

15 Date of submission: October 31, 2018

16 Number of figures: 5.

17 Word count: 6312.
18

19 **Running title:**

20 Determinant factors of stress-induced microspore embryogenesis in crops

ABSTRACT

Under stress, isolated microspores are reprogrammed *in vitro* towards embryogenesis, producing doubled haploid plants, useful biotechnological tools in plant breeding as a source of new genetic variability, fixed in homozygous plants in only one generation. Stress-induced cell death and low rates of cell reprogramming are major factors that reduce the process yield. Knowledge gained in recent years has revealed that microspore embryogenesis initiation and progression involve a complex network of factors, whose roles are not yet well understood. Autophagy and cell-death proteases are crucial players in the response to stress, while cell reprogramming and totipotency acquisition are regulated by hormonal and epigenetic mechanisms. Auxin biosynthesis, transport and action are required for microspore embryogenesis. Initial stages involve DNA hypomethylation, H3K9 demethylation, and H3/H4 acetylation. Cell wall remodelling, with pectin de-methylesterification and AGP expression, is necessary for embryo development. We will review recent findings regarding the determinant factors underlying stress-induced microspore embryogenesis, focusing on the role of autophagy, cell death, auxin, chromatin modifications, and cell wall. Recent reports show that treatments with small modulators of autophagy, proteases and epigenetic marks reduce cell death and enhance embryogenesis initiation in several crops, opening up new possibilities for improving *in vitro* embryo production in breeding programs.

INTRODUCTION

Plants, unlike animals, exhibit extraordinary developmental plasticity as they continuously form organs during post-embryonic development. The plasticity of plant cells, particularly their ability to regenerate embryos through *in vitro* culture, has been extensively exploited for decades for the purpose of plant propagation, breeding and conservation, with relevant applications in agriculture, forestry and the preservation of genetic resources (Germana and Lambardi, 2016). *In vitro* embryogenesis is a fascinating example of cellular totipotency, as different kinds of somatic cells can be reprogrammed giving rise to an entire embryo and ultimately a plant.

Microspore embryogenesis is an *in vitro* system in which the haploid microspore (pollen mother cell) is reprogrammed by the application of external stress treatments and enters into an embryogenesis pathway (Fig. 1). The resulting haploid embryo can diploidize either spontaneously or by application of chromosome doubling agents (Pintos *et al.*, 2007; Testillano *et al.*, 2004); Castillo *et al.* 2009), producing doubled haploid (DH) plants that are fully homozygous for each locus. DHs are important biotechnological tools in plant breeding mainly because they permit to considerably shorten the breeding process (Maluszynski *et al.*, 2003; Forster *et al.*, 2007; Murovec and Bohanec, 2012). With DH technology, completely homozygous plants can be established in only one generation while in a conventional breeding program the development of homozygous lines normally involves several generations of selfing and selection. DHs can be used as parental lines for hybrid production; they increase the selection efficiency since recessive alleles of improved characters are fixed and directly expressed in one generation. On the other hand, due to the homozygous condition of DHs, deleterious recessive alleles are eliminated at early steps of the breeding program (Prem *et al.*, 2004; Germaná 2011; Murovec and Bohanec 2012; Dwivedi *et al.*, 2015). Other advantage of DHs in crop breeding is the increase of the genetic gain; DH plants are regenerated from individual microspores and therefore, due to meiotic recombination, they contain new gene combinations which represent new recombinant products of the parental genomes fixed in homozygous state. Besides these advantages, DHs can be combined with other breeding approaches like mutation or gene transformation, leading to greatly accelerate cultivar development (Prem *et al.*, 2004; Gurushidze *et al.*, 2014; Dwivedi *et al.*, 2015; Ren *et al.*, 2017; Rustgi *et al.*, 2017). For

all these reasons, microspore embryogenesis *in vitro* systems are widely used by plant nursery and seed companies to rapidly generate new isogenic lines for breeding programs. Since 1964, the year in which the production of haploid embryos was first achieved from anther cultures of *Datura* (Guha and Maheshwari, 1964), *in vitro* systems of microspore embryogenesis have been developed for over 250 plant species belonging to a wide range of families (reviewed in (Maluszynski *et al.*, 2003), with variable efficiency. Even though the application of microspore embryogenesis to DH production is currently widely exploited, it is still highly, or even completely inefficient, in many species of economic interest in the fields of agriculture and forestry.

The yield of microspore-derived embryo production has several bottlenecks at various stages of the process, such as embryogenesis induction and initiation, which are both crucial steps. Induction of microspore embryogenesis is performed by application of specific stress treatments, such as cold, heat or starvation, using either anthers or isolated microspore cultures (Shariatpanahi *et al.*, 2006; Touraev *et al.*, 1996). After induction, the responsive microspores switch their developmental program from the gametophytic to the embryogenic pathway, while many other microspores die during the first days of *in vitro* culture (Fig. 1) (Maraschin *et al.*, 2005; Rodríguez-Serrano *et al.*, 2012; Satpute *et al.*, 2005). Efficient induction of microspore embryogenesis depends on multiple factors like genotype, donor plant condition, culture media composition and type of inductive stress applied. Besides these factors, the developmental stage of microspores is a critical parameter; the late vacuolated microspore stage (Fig. 2A) has been reported as the most responsive for embryogenesis induction in a wide range of plant species, including cereals and horticultural crops, as well as forest and fruit trees (Bueno *et al.*, 2003; Germana, 2009; Germanà *et al.*, 2011; González-Melendi *et al.*, 1995; Prem *et al.*, 2012; Solís *et al.*, 2008; Prem *et al.*, 2004). At the beginning of the process, the main limiting factors of microspore embryogenesis initiation are high levels of cell death and low reprogramming efficiency.

Stress-induced microspore embryogenesis is also an excellent *in vitro* system to study the regulating mechanisms of cell reprogramming, totipotency, cell fate decisions and early embryogenesis, since zygotes and immature embryos produced *in planta* are surrounded by maternal tissues and difficult to dissect. For decades, advances in plant *in vitro* protocols have been mostly based on trial-and-error approaches since the

mechanisms underlying the induction of dedifferentiation of a somatic cell and its reprogramming and conversion into a totipotent embryogenic cell remain elusive. A better understanding of the processes involved in the reprogramming induction and embryogenic competence acquisition will help to identify new targets and design new strategies to improve the efficiency of *in vitro* embryogenesis systems, even in recalcitrant species.

Two crop species, the monocot *Hordeum vulgare* (barley) and the eudicot *Brassica napus* (rapeseed), have been used as models for studying the cellular and molecular basis of microspore embryogenesis. In these two species, there are efficient and well-established *in vitro* systems of stress-induced microspore embryogenesis using isolated microspores cultures (Kumlehn *et al.*, 2006; Pechan and Keller, 1988; Prem *et al.*, 2012; Rodríguez-Serrano *et al.*, 2012). Anther culture, on the other hand, is especially useful in biotechnological applications of many herbaceous crops and some woody species, including several fruit and forest trees, in which long regeneration times and strong inbreeding depression make traditional breeding methods impractical (Jain *et al.*, 1996a, b, c, d, e; Touraev *et al.*, 2009). *Citrus clementina* is one of the best examples of a fruit tree in which the regeneration of double haploids by anther culture has been successful (Germana, 2009). Also, in cork oak (*Quercus suber* L.), successful microspore embryogenesis using anther cultures and embryo production protocols have been reported and later optimized (Bueno *et al.*, 1997) Testillano et al. 2018). After induction and culture initiation, responsive microspores divide and produce multicellular structures or proembryos, still confined within the microspore wall (exine) (Fig. 2B). Such structures are considered to be an early sign of embryogenesis initiation. As embryogenesis progresses, the exine breaks down, and embryos develop following a similar pathway to zygotic embryogenesis, producing globular, heart, torpedo (Fig. 2C) and cotyledonary embryos (Fig. 2D) in the case of dicot species, and globular, transitional, scutellar and coleoptilar embryos in the case of monocot plants. Microspore-derived embryos can germinate under appropriate culture conditions and further regenerate plants which can be acclimatized and growth in soil. Haploid plants growth normally and produce flowers that are completely sterile and often show malformations (Murovec and Bohanec 2012). Depending on the species, spontaneous diploidization can occur in a proportion of regenerated plants. In cereals, the rate of spontaneous diploidization is high, whereas in vegetables and trees it is much lower and

chemical treatments with drugs, such as colchicine, oryzalin, amiprophosmethyl, trifluralin or pronamide, should be applied during DHs production (Jain *et al.*, 1996a; Barnabás *et al.*, 1999; Pintos *et al.*, 2007; Murovec and Bohanec, 2012). The frequency of spontaneous genome doubling has been reported up to 40-50% in *Brassica napus*, 40% in maize, 60% in rice, and 90% in barley and rye (Castillo *et al.*, 2009; Prem *et al.*, 2012; Ren *et al.*, 2017), with nuclear fusion as the main mechanism for spontaneous diploidization in cereal species (Kasha *et al.*, 2001, 2005; González-Melendi *et al.*, 2005; Testillano *et al.*, 2004).

Our understanding of the cellular and molecular basis of microspore embryogenesis induction has improved in recent years; however, the mechanism underlying the change in microspore cell fate is still largely unknown. Advances in this area have been hampered by several factors such as: (1) the difficulty involved in applying genetic approaches genotypes that are responsive to embryogenesis cannot be efficiently transformed, lack of transformation procedures and mutants in many crop species, etc.); (2) recalcitrance of the model plant *Arabidopsis* to microspore embryogenesis induction; and (3) the difficulty of dissecting early developmental stages of the process for analyses by molecular and biochemical approaches. After induction, a high proportion of non-responsive microspores coexist in the *in vitro* culture with a much lower percentage of responsive microspores that start to divide producing multicellular microspores/proembryos, and separation of these early embryogenic structures is technically very difficult at the onset of embryogenesis initiation. Cellular approaches using *in situ* molecular identification techniques have provided unique information by localizing key molecules and differential gene expression in microspore embryogenic structures from the very initial stages, by advanced imaging microscopy technologies (Rodríguez-Sanz *et al.*, 2015; Testillano and Risueño, 2009; Testillano and Rodríguez, 2012) — methodologies that permit very early embryogenic structures (2–3 cells) to be distinguished from non-embryogenic ones. Changes in various cell activities and rearrangements in the structural organization of subcellular compartments have been shown to accompany the microspore reprogramming process in several herbaceous and woody species (Telmer *et al.*, 1995; Bárány *et al.*, 2005; Seguí-Simarro *et al.*, 2006; Solís *et al.*, 2008; Testillano *et al.*, 2000; Testillano *et al.*, 2002), and some common features in the developmental pattern among species have been identified, as have

species-specific features of woody plants (Chiancone *et al.*, 2015; Rodriguez-Sanz *et al.*, 2014a).

Here, we will review recent findings regarding the determinant factors that underlie stress-induced microspore embryogenesis induction and progression, with special emphasis on the role of autophagy, cell death, endogenous auxin, chromatin modifications, and cell wall remodelling. Information gained over the last few years has also shown that the modulation of these determinant factors by small bioactive molecules affects the process yield, emerging as a promising strategy to improve microspore embryogenesis efficiency (Barany *et al.*, 2018; Berenguer *et al.*, 2017; Solis *et al.*, 2015); Pérez-Pérez *et al.*, 2018a). This has opened up a completely new intervention pathway to increase the efficiency of the *in vitro* production of embryos and doubled-haploid plants in crop and forest species, especially recalcitrant ones, with the aim being their application in breeding programs.

STRESS-INDUCED CELL DEATH, AUTOPHAGY AND PROTEASES

Stress treatments are necessary to produce the embryogenic response of microspores. Cold and mild heat treatments are the most frequently used conditions to induce microspore embryogenesis in many plant species (Maluszynski *et al.*, 2003; Shariatpanahi *et al.*, 2006). In the model systems *B. napus* (rapeseed) and *H. vulgare* (barley), the inductive stress treatments are 32°C and 4°C, respectively. The expression of some stress-related proteins, such as heat-shock proteins HSP70 and HSP90, has been reported in microspore embryogenesis cultures of *B. napus* and *Capsicum annum* (Cordewener *et al.*, 1995, 2000; Seguí-Simarro *et al.*, 2003; Bárány *et al.*, 2001); although a protective role for these chaperones in response to stress has been suggested, their role in microspore embryogenesis is not clear.

Analyses of cell viability have shown that there is a marked increase in cell death levels after stress treatment in several microspore embryogenesis systems (Pérez-Pérez *et al.* 2018a, (Rodríguez-Serrano *et al.*, 2012). The inductive stress for microspore embryogenesis also leads to oxidative stress, with the production of reactive oxygen species (Rodríguez-Serrano *et al.*, 2012; Zur *et al.*, 2009). The balance between ROS-

producing and ROS-scavenging endogenous mechanisms determines the level of oxidative stress and damage in the cell, leading to cell death when it surpasses a certain threshold (Clarke *et al.*, 2000). The ability to control oxidative stress to prevent cellular oxidative damage and maintain cell homeostasis is a key factor for stress-induced microspore embryogenesis. In this sense, several enzymes of the anti-oxidative machinery of the cell have been found to increase their activity/expression in microspore cultures, with a protective role being suggested for these stress-related proteins (Maraschin *et al.*, 2006; Muñoz-Amatriain *et al.*, 2006; Zur *et al.*, 2009; Uváčková *et al.*, 2012).

Autophagy, which is a major catabolic pathway for recycling cell materials, has many different functions under stress conditions or during specific developmental processes (reviewed in (Avin-Wittenberg *et al.*, 2018; Hofius *et al.*, 2017; Masclaux-Daubresse *et al.*, 2017). In plants, autophagy has been shown to have a role in promoting cell survival under starvation and stress conditions, as well as in cell death initiation and/or execution (Avin-Wittenberg, 2018; Avin-Wittenberg *et al.*, 2018; Minina *et al.*, 2013; Minina *et al.*, 2014). Increasing evidence has connected ROS and autophagy in plants and algae (Perez-Perez *et al.*, 2012). Induction of autophagy has been shown in response to ROS treatments (Perez-Perez *et al.*, 2010). A recent study on barley microspore embryogenesis reported the activation of autophagy after the inductive stress at 4°C (Barany *et al.*, 2018). Stress-treated microspores showed up-regulation of autophagy genes *HvATG5* and *HvATG6*, and an increase in the number of autophagosomes which were labelled by ATG5 and ATG8 antibodies (Fig. 3) and exhibited the typical ultrastructure of autophagosomes under electron microscopy (Barany *et al.*, 2018).

The plant proteolytic machinery comprises a large number of proteases that regulate cell homeostasis, and many of them have been shown to play key roles in both autophagy and cell death processes (Van Der Hoorn and Jones, 2004; van der Hoorn and Rivas, 2018). Papain-like C1A cysteine proteases (PLCPs or cathepsins) are the most abundant enzymes with a role in plant senescence, programmed cell death (PCD), and proteolysis mediated by stress (Diaz-Mendoza *et al.*, 2016; Velasco-Arroyo *et al.*, 2016). In animals, cathepsins are well known lysosomal proteases with a role in autophagy and cell death (Turk and Stoka, 2007), although in plants PLCP function in autophagy has not yet been demonstrated directly. Studies on cell death-related proteases involved in

the stress response of barley microspores have revealed that cathepsin L/F-, B- and H-like activities were induced after stress, and cathepsin-like genes *HvPap-1* and *HvPap-6* were up-regulated (Barany *et al.*, 2018). Caspases are another important group of cell death proteases. Despite the fact that plant genomes do not contain structural homologues of caspases, caspase-like activities have been detected in many plant species, and are required for PCD execution (Minina *et al.*, 2017). Plant metacaspases constitute a major protease group of the large C14B family of proteases with relevant roles in PCD and autophagy (Minina *et al.*, 2017; Minina *et al.*, 2013). Preliminary results regarding *B. napus* microspore embryogenesis have revealed that the stress treatment of 32°C, used to induce embryogenesis, leads to autophagy activation, induction of metacaspase enzymatic activity and gene expression (Berenguer *et al.* 2018).

Interestingly, pharmacological treatments with inhibitors of ROS (MnCl₂, Ascorbate), autophagy (3-MA, concanamycin A) and protease activities, particularly PLCP, caspase 3-like, and metacaspase activities (E64, Ac-DEVD-CHO, Ac-VRPR-FMK), lead to the reduction of cell death levels, and consequently an increase in the embryogenesis initiation rate in stress-induced microspore embryogenesis cultures of rapeseed and barley (Barany *et al.*, 2018), Pérez-Pérez *et al.* 2018a). These findings reveal the implication of autophagy in cell death of microspores after the inductive stress, as well as the participation of cathepsin and caspase 3-like proteolytic activities. Further work will be necessary to explore whether autophagy can also play a role promoting cell viability in response to stress in a certain population of microspores, as is the case in other plant cells (Avin-Wittenberg, 2018), a possibility that cannot be ruled out. These findings also open up new intervention pathways to reduce stress-induced cell death levels at early stages of microspore embryogenesis by modulating autophagy and protease activities.

ENDOGENOUS AUXIN

Among plant growth regulators, auxin is the most significant hormone in plant development and it is the key regulator of cell division and differentiation (Teale *et al.*, 2006; Weijers *et al.*, 2018). The induction of most somatic embryogenesis systems,

from cells other than microspores, is mostly triggered by exogenous hormones, and this is commonly achieved using the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), as well as via stress treatments (Feher, 2015). On the contrary, the majority of microspore embryogenesis systems do not require exogenous auxin as an inducer, whereas a transient physical (thermal) or chemical stress is essential to trigger the change of developmental cell fate, probably as a response guided by endogenous hormones. In the case of anther culture, some protocols include growth regulators in their media composition. In this sense, microspore embryogenesis using isolated microspore cultures represents a convenient system for analysing endogenous hormone function during *in vitro* embryogenesis initiation and progression, since the process is induced and the embryo is differentiated *in vitro* from individual isolated cells without the addition of auxin or other hormones at any stage.

Auxin action depends on its local biosynthesis and differential distribution within plant tissues, mainly regulated by its polar transport among cells (Petrasek and Friml, 2009). Different pathways leading from auxin perception to gene expression and to non-transcriptional responses have been identified in recent decades, although many aspects of the complex mechanisms of signalling and action involved remain elusive (Weijers *et al.*, 2018). The key components of auxin biosynthesis, transport, and signalling have been elucidated (review in (Li *et al.*, 2016; Mironova *et al.*, 2017); among them, the genes of auxin biosynthesis *TAA1* and *YUC*, the efflux carrier *PIN1*, and the auxin response factors, *ARFs*, are all crucial elements in the control of auxin action (Li *et al.*, 2016). During zygotic embryogenesis *in planta*, the major form of auxin, indole acetic acid (IAA), has demonstrated key functions, particularly in embryo patterning, polarization and differentiation (Moller and Weijers, 2009; Rademacher *et al.*, 2012).

Several studies have shown endogenous auxin accumulation in early microspore embryo cells of *B. napus* and *Quercus suber* (Prem *et al.*, 2012; Rodriguez-Sanz *et al.*, 2015), as well as in *H. vulgare* (El-Tantawy 2016); other studies have localized auxin response in microspore embryos of *B. napus* (Dubas *et al.*, 2014; Soriano *et al.*, 2014) by expression of the DR5-auxin reporter. In addition, various reports have documented that abiotic stress factors can impact on auxin homeostasis, leading to a wide range of plant cell responses (da Costa *et al.*, 2013; Potters *et al.*, 2007; Tognetti *et al.*, 2012). Interactions between stress signalling and hormones, particularly auxin, have been

proposed to lead to somatic embryogenesis induction (Feher, 2015). Recent studies have revealed the involvement of endogenous auxin in microspore reprogramming and *in vitro* embryo formation in *B. napus*. After microspore embryogenesis induction, *de novo* endogenous auxin biosynthesis and early accumulation of IAA in proembryo cells are detected, from the first embryogenic divisions (Rodriguez-Sanz *et al.*, 2015). Moreover, *BnTAA1* and *BnPIN1* expression are both up-regulated throughout microspore embryogenesis initiation and progression, correlating with the increase in IAA concentration. Pharmacological treatments with inhibitors of auxin action, PCIB, polar transport, NPA (Rodriguez-Sanz *et al.*, 2014a; Rodriguez-Sanz *et al.*, 2015), and biosynthesis, kynurenin (Pérez-Pérez and Testillano, unpublished) severely disturb microspore embryogenesis initiation and progression, resulting in a significant reduction in embryo production yield (Rodriguez-Sanz *et al.*, 2015). These findings clearly indicate that auxin biosynthesis, activity and transport are required for stress-induced microspore embryogenesis.

Together with auxin, cytokinin is a key regulator of plant growth and development. The balance of these two hormones, which usually act antagonistically, controls cell division and differentiation. In recent years, several studies have revealed the interrelationship of auxin and cytokinin signalling pathways, and the importance of their concentration balance and antagonistic effects as key factors for cell fate, proliferation and differentiation (Perianez-Rodriguez *et al.*, 2014). In comparison with auxin, less is known about the biosynthesis, signalling pathway and transport of cytokinins (Duran-Medina *et al.*, 2017). Recent studies have shown that establishment of the spatiotemporal distribution of auxin and cytokinin response signals is central for the control of early somatic embryogenesis and meristem initiation in *Arabidopsis* (Su *et al.*, 2014). Supplementation with exogenous cytokinins has been tested in some anther culture systems with variable results (Zur *et al.*, 2015); however, the involvement of endogenous cytokinin in microspore embryogenesis remains to be analysed. Preliminary results of our group show that auxin and cytokinin have opposite patterns of distribution in microspore-derived developing embryos, suggesting a defined spatiotemporal localization of auxin and cytokinin responses during microspore embryogenesis.

Further work will be necessary to explore the hormonal crosstalk and signalling pathways that regulate microspore embryogenesis induction, and to determine how the inductor stress signalling interacts with auxin and cytokinin signalling pathways to drive the switching of cell fate.

EPIGENETIC MODIFICATIONS AND CHROMATIN REMODELLING

The reprogramming of somatic cells and embryogenesis initiation involve genome-wide changes of gene expression that allow the old cell fate program to be halted and the activation of a new developmental program. These changes in gene expression are characterized by the change in global genome organization and the remodelling of chromatin, in both plants and animals (Kouzarides, 2007). Increasing evidence has indicated the involvement of epigenetic mechanisms in the regulation of plant *in vitro* morphogenic processes, including organogenesis and somatic embryogenesis (Feher, 2015; Miguel and Marum, 2011), as well as in response to environmental cues and to stress (Gutzat and Mittelsten Scheid, 2012; Luo *et al.*, 2012). DNA methylation and histone modifications, mainly methylation and acetylation, are the most important epigenetic marks controlling chromatin organization.

DNA methylation constitutes a prominent epigenetic modification of the chromatin fibre which is locked in a transcriptionally inactive conformation leading to gene silencing (Wang and Kohler, 2017). Commonly, open chromatin increases the accessibility of the genome to the transcription machinery, while closed chromatin represses gene expression by limiting accessibility (Kouzarides, 2007; Reyes, 2006). Several reports have related totipotency of cells to an open chromatin conformation characterized by large nuclei and homogenous euchromatin (Grafi *et al.*, 2011). During microspore embryogenesis, changes in global DNA methylation levels have been observed in various herbaceous and woody species (Corredoira *et al.*, 2017; El-Tantawy *et al.*, 2014; Rodriguez-Sanz *et al.*, 2014b; Solis *et al.*, 2012; De-la-Peña *et al.*, 2015; Li *et al.*, 2016). Using quantitative biochemical assays and immunolocalization of 5-deoxy-methyl-cytosine (5mdC), these studies demonstrated that microspore reprogramming and embryogenesis initiation involve a global DNA hypomethylation.

The change in developmental program and initiation of embryogenesis affected the functional organization of the nuclear domains, including chromatin condensation state (Seguí-Simarro *et al.*, 2006; Seguí-Simarro *et al.*, 2011; Testillano *et al.*, 2000; Testillano *et al.*, 2005; De-la-Peña *et al.*, 2015). After induction, early microspore proembryos are characterized by a decondensed chromatin pattern that exhibits low DNA methylation, as revealed by 5mdC labelling (El-Tantawy *et al.*, 2014; Solis *et al.*, 2012). In contrast, further embryo development is characterized by a progressive increase in global methylation of embryo cells, associated with the heterochromatization that accompanies cellular differentiation. In *B. napus*, gene expression of the DNA methyltransferase *MET1* is up-regulated during microspore embryogenesis progression, as well as in advanced stages of zygotic embryogenesis, suggesting that MET1 activity contributes to the increase in global DNA methylation of differentiating embryo cells (Solis *et al.*, 2012). Experiments with modulation of global DNA methylation levels using inhibitors of methylation, like 5-azacytidine (azaC) or 5-aza-2'-deoxycytidine (azadC), have been used in various *in vitro* systems of somatic embryogenesis and organogenesis, with variable results due to high dose response effects and cell toxicity. However, when azaC or azadC are applied at early stages and at a low concentration, they produced DNA hypomethylation and promoted embryogenesis initiation, as reported in Arabidopsis somatic embryogenesis with azadC (Elhiti *et al.*, 2010) and in microspore embryogenesis of *B. napus* and *H. vulgare* with azaC (Solis *et al.*, 2015). The reduction of global DNA methylation by azaC treatment during advanced embryogenesis stages results in an impairment of embryo development and indicates that de novo DNA methylation is required for microspore embryogenesis progression (Solis *et al.*, 2015). The way in which differentiating plant cells reprogram and acquire cell totipotency is a central question that involves remodelling of genome-wide expression programs and large-scale chromatin reorganization. In this regard, these recent findings support the idea that DNA hypomethylation is critical for the regulation of chromatin remodelling and the switching of the gene expression program in microspore embryogenesis induction.

Together with DNA methylation, histone modifications—mainly methylation and acetylation—participate by driving changes in chromatin conformation and condensation (Eichten *et al.*, 2014; Yang *et al.*, 2010), controlling gene expression during plant development and in response to the environment. Chromatin-modifying

enzymes, including histone lysine methyltransferases (HKMTs) and demethylases (LSD1 and JmjC families), as well as histone acetyltransferases (HATs) and deacetylases (HDACs), have all been proposed as modulators of cell reprogramming that act by changing the genome-wide distribution of repressive and permissive histone marks and promoting open/closed chromatin states (Onder *et al.*, 2012). Methylation of histones can occur on different lysine residues in histones H3 and H4. H3 methylation at positions K9 and K27 is generally related to gene silencing, while active genes are associated with methylation at K4 and K36 (Liu *et al.* 2010). Among these epigenetic marks, H3K9 methylation is one of the major histone modifications with central roles in the epigenetic control of diverse developmental processes. In plants, H3K9 methylation is associated with DNA methylation and small RNAs, which are both essential drivers for heterochromatin formation (Saze *et al.*, 2012). H3K27 methyltransferases of POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) have been associated with the prevention of pluripotency during differentiation in *Arabidopsis* (Feher, 2015; Gentry and Hennig, 2014; Gu *et al.*, 2014; She *et al.*, 2013), and PRC2 activity has been reported to block hormone-mediated reprogramming towards somatic embryogenesis in *Arabidopsis* (Mozgova *et al.*, 2017).

A recent report has shown that microspore reprogramming and the initiation of embryogenesis are associated with low levels of H3K9 methylation, while embryo differentiation progresses with increasing levels of this histone mark (Berenguer *et al.*, 2017; Rodriguez-Sanz *et al.*, 2014b), as demonstrated by biochemical and H3K9me2 immunofluorescence assays (Fig. 4). Interestingly, the temporal profile of bulk H3K9 methylation during microspore embryogenesis correlates with the gene expression patterns of *BnHKMT SUV4-like* and *BnLSD1-like* genes, ‘writer’ and ‘eraser’ enzymes of H3K9me2 (Berenguer *et al.*, 2017), providing additional evidence of the developmental regulation of this histone modification during the process of microspore embryogenesis. Moreover, treatment of microspore cultures with the small molecule BIX-01294, a known inhibitor of H3K9 methylation in mammalian cells (Kubicek *et al.*, 2007; Tachibana *et al.*, 2002), promotes microspore reprogramming and embryogenesis initiation, in rapeseed and barley (Berenguer *et al.*, 2017). Conversely, BIX-01294 treatment at advanced stages impairs embryo formation, showing that embryogenesis initiation involves low H3K9 methylation but embryo differentiation requires high levels of this repressive mark.

It has been shown that the acetylation of lysine residues within the N-terminal tail of histones H3 and H4 is also involved in microspore embryogenesis initiation and progression, and increasing acetylation has been related to cell totipotency (Li *et al.*, 2014a; Rodriguez-Sanz *et al.*, 2014b). Histone H3 and H4 acetylation is mainly associated with actively transcribed genes and promotes the open chromatin states (Onder *et al.*, 2012). The application of histone deacetylase inhibitors —like trichostatin A or SAHA, which lead to increased levels of histone acetylation— produce an increase in the rate of microspore embryogenesis induction (Zhang *et al.* 2016; (Jiang *et al.*, 2017; Li *et al.*, 2014a; Pandey *et al.*, 2017) Berenguer *et al.*, in preparation), demonstrating that histone acetylation plays a crucial role in promoting microspore reprogramming and totipotency.

Increasing evidence points to the existence of a complex crosstalk between DNA methylation and histone methylation/acetylation in the regulation of plant developmental processes (Saze *et al.*, 2012). Evidence obtained to date shows that a genome-wide chromatin remodelling is required for microspore reprogramming, totipotency and embryogenesis initiation; global hypomethylation of DNA and H3K9, and increasing histone acetylation would presumably mainly contribute to this chromatin remodelling by increasing both chromatin decondensation and the access of transcription factors regulating the switch of the developmental program.

CELL WALL REMODELLING: PECTINS AND AGPS

A growing number of studies support the crucial role of cell wall components such as pectins and arabinogalactan proteins (AGPs) during somatic and zygotic embryogenesis in plants (El-Tantawy *et al.*, 2013; Rodriguez-Sanz *et al.*, 2014a; Samaj *et al.*, 2005; Van Hengel *et al.*, 2002). Plant cell walls are dynamic and complex structures that play important roles in the regulation of plant growth, development, and the determination of cell shape and fate (Somerville *et al.*, 2004). Modifications in cell wall components have been reported as being crucial in relation to cell fate and development. Plant growth and differentiation requires controlled remodelling of cell wall polymer networks, producing changes in their mechanical properties to permit cell division and expansion (Baluska *et al.*, 2002; Baluska *et al.*, 2005; Geshi *et al.*, 2013; Samaj *et al.*,

2005; Seifert and Roberts, 2007; Smertenko and Bozhkov, 2014; van Hengel *et al.*, 2001; Van Hengel *et al.*, 2002). Cell differentiation requires remodelling of wall polysaccharide networks during development and in response to external signals (Barnes and Anderson, 2018). Among cell wall polymers, pectins are major components of the primary wall and represent one of the most complex families of polysaccharides. Pectins are exported to the cell wall in a highly methylesterified form and their level of methylesterification is controlled *in muro* by pectin methylesterases (PMEs) and pectin methyl esterase inhibitors (PMEI) (Pelloux *et al.*, 2007). The de-methylesterified residues of pectins can form Ca²⁺ bonds which may promote cell wall strengthening or become a target for pectin-degrading enzymes, such as polygalacturonases, affecting the texture and rigidity of the cell wall (Pelloux *et al.*, 2007). The change in the developmental program of the microspore and the progression of embryo development involve changes in pectin esterification that are associated with proliferation and differentiation events, as demonstrated by highly sensitive immunocytochemical and immunodot-blot assays with specific antibodies (Bárány *et al.*, 2010a, b; Solis *et al.*, 2016). These modifications in the esterification status of pectins may cause the cell wall remodelling during the process. At early embryogenic stages, high levels of methylesterified pectins have been found (Fig. 5A, B) as a differential feature of the cell wall after cell reprogramming in the stress-induced microspore embryogenesis of several species (Corredoira *et al.*, 2017; Rodriguez-Sanz *et al.*, 2014a; Solis *et al.*, 2016; Solís *et al.*, 2008). As embryogenesis proceeds, decreasing methylesterification levels have been reported in cell walls of differentiating embryos, in agreement with observations in other developmental pathways from proliferating to differentiating tissues of various plant species (Jolie *et al.*, 2010). Changes in pectin esterification levels correlate with temporal expression patterns of *PME* and *PMEI* genes in microspore embryogenesis of *B. napus* (Solis *et al.*, 2016), and unpublished results) and somatic embryogenesis of *Q. suber* (Pérez-Pérez *et al.* 2018b). For example, differentiating embryos (like torpedo and cotyledonary embryos) show cell walls rich in methylesterified pectins, together with high expression levels of *PME* (Fig. 5C, 5D). Moreover, inhibition of PME activity by catechin (Lewis *et al.*, 2008) impairs somatic embryogenesis in *Q. suber* (Pérez-Pérez *et al.* 2018b), indicating that pectin esterification and cell wall configuration play a relevant role in embryogenesis induction and/or progression (Solis *et al.*, 2016), Pérez-Pérez *et al.* 2018b). Taken together, these findings support the idea that PME-mediated configuration of pectins

could be a crucial factor for microspore embryo differentiation, acting via the promotion of cell wall remodelling during the process. Auxin has been related to the regulation of cell wall remodelling during initiation of organogenesis in *Arabidopsis*, exerting its effect by reducing the cell wall stiffness through a process that requires de-methylesterification of pectins (Braybrook and Peaucelle, 2013; Lewis *et al.*, 2013). Further work will be necessary to determine the exact role of auxin in pectin-related cell wall remodelling during stress-induced microspore embryogenesis.

Plant cell walls also contain structural proteins such as arabinogalactan proteins (AGPs). AGPs are a large family of highly glycosylated hydroxyproline-rich proteins that are present in cell walls, plasma membranes and extracellular secretions. Various lines of evidence have shown that AGPs play key roles in many plant developmental processes, including embryo development (Geshi *et al.*, 2013; Seifert and Roberts, 2007; Zhong *et al.*, 2011). They have also been proposed as modulators of cell wall mechanics (Seifert and Roberts, 2007). Many AGPs contain a glycosylphosphatidylinositol (GPI) anchor, which tethers them to the plasma membrane and allows them to be positioned over the surface of the plasma membrane and the cell wall (Seifert and Roberts, 2007). Moreover, like other signalling molecules, AGPs can also be exported into the cell wall, with them then being spread to the surrounding cells and secreted into the culture medium. Supplementing culture medium with exogenous AGPs (commonly Arabic gum containing an AGP mixture) promotes somatic embryogenesis in several plant species (Smertenko and Bozhkov, 2014; Thompson and Knox, 1998; Yuan *et al.*, 2012). The secretion of endogenous AGPs from cultured cells into medium has been shown to stimulate embryo development in microspore and zygote cultures of maize (Borderies *et al.*, 2004; Testillano *et al.*, 2010), carrot embryogenic suspension cultures (van Hengel *et al.*, 2001), and somatic embryogenesis cultures of cotton (Poon *et al.*, 2012). Several reports have also shown the differential presence of AGPs in various *in vitro* embryogenic systems (Samaj *et al.*, 2005) and during microspore embryogenesis (Malik *et al.*, 2007; El-Tantawy *et al.*, 2013). The localization of AGPs in cell walls and secretory vesicles has also been demonstrated, as revealed by immunofluorescence with a battery of highly specific monoclonal antibodies against AGP epitopes (Knox, 1997) at early stages of microspore embryogenesis (El-Tantawy *et al.*, 2013). Information regarding the expression of AGP genes in *in vitro* embryogenesis is very scarce. Some studies have shown up-regulation of some AGP

genes associated with the initiation of somatic embryogenesis in *Picea balfouriana* (Li *et al.*, 2014b) and microspore embryogenesis in *B. napus* (Malik *et al.*, 2007; El-Tantawy *et al.*, 2013).

Studies with Yariv reagents (Yariv *et al.*, 1967; Yariv *et al.*, 1962), which block AGPs have revealed that the inactivation of AGPs inhibited embryogenesis in several *in vitro* systems and in microspore embryogenesis cultures of *B. napus* (Tang *et al.*, 2006). AGPs can be trapped by pectins and may modulate the mechanical properties of the pectic matrix (Liao *et al.*, 2011; Seifert and Roberts, 2007). AGPs are up-regulated and required in microspore embryogenesis; they might contribute to cell wall remodelling either by structural interaction with pectins or other wall polymers, or by directly stiffening the cell wall by oxidative crosslinking—a proposed mechanical role for AGPs in cell walls (Seifert and Roberts, 2007). These findings indicated a role for both pectins and AGPs in the cell wall remodelling that takes place and is required in microspore embryogenesis, with pectin de-esterification and AGP expression being necessary for embryo formation.

CONCLUDING REMARKS

The knowledge gained in recent years has revealed that microspore embryogenesis initiation and progression involve a complex network of determinant factors, whose roles are not yet well understood. Stress-induced cell death and low rates of cell reprogramming are major factors that greatly reduce the process yield at the initial stages. Autophagy and cell-death proteases (metacaspases and cathepsins) have emerged as crucial players in the response of microspores to the inductive stress, leading to cell death, while cell reprogramming and totipotency acquisition of responsive microspores are mainly regulated by hormonal and epigenetic mechanisms. Activation of endogenous auxin biosynthesis, as well as its polar transport and action, probably in combination with an appropriate cytokinin/auxin balance, are required for microspore embryogenesis, with initial stages involving global epigenetic changes such as hypomethylation of DNA, H3K9 demethylation, and H3/H4 acetylation. Supplementing the culture medium with specific modulators that induce these global epigenetic changes (such as azaC, azadC, BIX-01294, Trichostatin A or SAHA) leads to

higher rates of embryogenesis initiation, indicating the crucial role of epigenetic reprogramming in microspore embryogenesis induction. Furthermore, after embryogenesis induction, the remodelling of the cell wall—with increasing pectin demethylesterification and AGP expression—is necessary for embryo development. Increasing evidence has revealed the involvement of auxin in signalling processes of chromatin remodelling by epigenetic mechanisms leading to the activation of specific gene expression programs. Additionally, auxin has been reported to control cell wall remodelling during various plant developmental processes. All these factors might be interconnected in the regulation of microspore reprogramming, embryogenesis initiation and progression, although future studies will be necessary to determine the signalling pathways involved.

Recent reports of pharmacological treatments with autophagy, proteases and epigenetic modulators are very promising in terms of enhancing *in vitro* embryo production yield. These modulators have shown similar beneficial effects in stress-induced microspore embryogenesis in various crop species, monocots and eudicots, independently of the inductive stress applied (cold or heat), which suggests that common mechanisms may operate in other plants and that a similar pharmacological strategy could be extended to other species to increase the microspore viability, reprogramming and embryogenesis induction rate. Recently, rapid advances have been reported in mammalian cell reprogramming by using small bioactive molecules which have been demonstrated to efficiently induce reprogramming and formation of pluripotent stem cells for various therapeutic applications (review in (Ma *et al.*, 2017). Although the mechanisms by which these small compounds induce reprogramming are unknown, they have shown activity as epigenetic modulators, autophagy modulators and inhibitors of various signalling pathways. The potential of the screening of chemical libraries of small molecules in plant biology research has been reported, although its efficient application in plants is still quite limited (Chuprov-Netochin *et al.*, 2016; Hicks and Raikhel, 2012). Furthermore, increasing evidence has revealed that stem cells in plants and animals behave similarly (Olariu *et al.*, 2017).

The recent findings reported here support the hypothesis that treatments with modulators of the recently identified key regulating processes—or with novel small molecules with reported cell reprogramming activity—will increase the efficiency of *in*

in vitro microspore embryogenesis, by reducing cell death levels and enhancing cell reprogramming and embryogenesis initiation. Future studies to identify new elements of the regulatory hormonal and epigenetic pathways controlling plant cell reprogramming, together with the screening of chemical libraries of novel small bioactive molecules (including autophagy, epigenetic and enzymatic activity modulators) will pave the way for new biotechnological strategies, by using small cell-permeable synthetic molecules—which are easy to apply and remove from cultures—to enhance cell reprogramming. This will open new possibilities for improving the efficiency of *in vitro* microspore embryogenesis systems for doubled haploid plant production in breeding and conservation programs, even in recalcitrant crops.

ACKNOWLEDGEMENTS

Research at Testillano's laboratory is supported by projects (AGL2014-52028-R and AGL2017-82447-R) funded by Spanish Ministry of Economy and Competitiveness (MINECO) and European Regional Development Fund (ERDF/FEDER). Support of TRANSAUTOPHAGY COST action, European Network of Multidisciplinary Research and Translation of Autophagy Knowledge (CA15138), is acknowledged. The author thanks to M.C. Risueño for helpful scientific discussions, and M.T. Solís, I. Bárány, E. Berenguer, Y. Pérez-Pérez, E. Carneros and A. Calvo for their contributions to the figures.

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FIGURE LEGENDS

Figure 1: Schematic representation of the two developmental pathways of the microspore. *In vivo*, the vacuolated microspore divides asymmetrically giving rise to the bicellular pollen grain, which follows the gametophytic development and produces the tricellular pollen. *In vitro*, by the application of specific stress treatments, the vacuolated microspore is reprogrammed towards an embryogenic pathway, initially producing proembryos (multicellular structures confined by the microspore wall) and later fully differentiated embryos, able to germinate and produce doubled-haploid plants. In this *in vitro* pathway, some microspores do not respond to reprogramming and die, after the stress.

Figure 2: Main stages of microspore embryogenesis in *Brassica napus*. A: Vacuolated microspore at the beginning of the culture. B: Proembryo formed after a few days (4-6 days) in culture, by several divisions of the microspore. D: Globular, heart and torpedo embryos developed from microspore cultures after 15-20 days in culture. E: Cotyledonary embryos completely differentiated after 30 days in culture. A, B: Micrographs of resin sections stained by toluidine blue. C, D: Views from stereomicroscope of embryos in Petri dishes of microspore cultures. Bars in A, B: 20µm; in C, D: 1mm.

Figure 3: Autophagy induction in stress-induced microspore embryogenesis of *Hordeum vulgare*. Confocal microscopy merged images of localization of ATG8 protein in autophagosomes (green) by immunofluorescence with ATG8 antibodies (Abcam) and nuclei stained with DAPI (blue). A: Microspore before the stress treatment, with no ATG8 signal. B: Stress-treated microspore showing ATG8 labelling on numerous small cytoplasmic spots, corresponding to autophagosomes. Bars: 20µm.

Figure 4: Localization of H3K9me2 during microspore embryogenesis of *Hordeum vulgare*. Confocal microscopy images of H3K9me2 immunofluorescence (green, A', B', C') and DAPI-stained nuclei (blue, A, B, C). Pairs of images A-A', B-B' and C-C' correspond to the same structures. A-A': Vacuolated microspore before induction showing mid intense labelling on the nucleus. B-B': Proembryo with several large

and rounded nuclei exhibiting lower immunofluorescence for H3K9me2. C-C':
Region of a cotyledonary embryo with numerous nuclei intensely labelled by
H3K9me2 antibodies. Bars in A, A', B, B': 20µm; in C, C': 75µm.

Figure 5: Changes in pectin methylesterification of cell wall during microspore
embryogenesis in *Brassica napus*. Confocal microscopy merged images of
immunofluorescence (A, B) or FISH, fluorescence *in situ* hybridization (C, D)
(green) and DAPI-stained nuclei (blue). A, B: Proembryos, early stage after
embryogenesis induction. A: Localization of highly esterified pectins (by JIM7
antibody) showing intense signal on cell walls of proembryos. B: Localization of de-
esterified pectins (by JIM5 antibody), cell walls of proembryos show much less
immunofluorescence signal. C: FISH of *BnPME1* in torpedo embryo showing an
intense signal corresponding to high expression in cytoplasm. D: Negative control
of FISH assay with the sense probe, in torpedo embryo. Bars: 20µm.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5