Microspore embryogenesis: reprogramming cell fate from pollen to embryo development

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Chapter 1

The Application of Microspore Embryogenesis for Plant Breeding and Fundamental Studies

1. Introduction

Microspore embryogenesis is a process in which immature male gametophytes are induced to switch from gametophyte to embryo development during *in vitro* culture. These haploid embryos can be converted into homozygous doubled-haploid (DH) plants, either spontaneously or after treatment with chromosome doubling agents. DH technology allows plant breeders to produce homozygous plants in a single generation and is used to accelerate plant breeding programs. Microspore embryogenesis also offers an alternative system to study embryo development and plant cell totipotency without the interference of maternal tissue. In this introduction, I discuss both applied and fundamental aspects of microspore embryogenesis, and where it is relevant and possible, put this knowledge in the context of zygotic embryogenesis and other *in vitro* embryo systems. A review of the early cellular and molecular events involved in the establishment of microspore embryo development is presented in Chapter 2 (Soriano et al., 2013).

2. Basic requirements for microspore embryo induction

The first *in vitro* haploid embryo culture system was developed around 50 years ago in *Datura innoxia* using anther culture (Guha and Maheshwari, 1964). Later, Nitsch (1974) cultured *Nicotiana* microspores released from anther tissue ('shed microspore' culture). In 1982, Lichter (1982) established the isolated microspore culture system from *Brassica* flower buds. In anther/shed microspore culture, whole immature anthers are cultured, while in microspore culture, the immature male gametophytes (microspores and pollen) are isolated and somatic anther tissue is removed prior to culture. Compared to anther culture, microspore culture has some advantages: (1) isolated microspore develop without interference from the anther tissue, the anther wall may have negative impact on embryo formation, or may produce diploid somatic embryos (Heberle-Bors, 1985); (2) the isolated microspores/pollen take up nutrients directly from the culture medium; (3) microspore culture is generally more efficient than anther culture, as it does not require anther dissection, but rather relies on bulk mechanical procedures to release the microspores/pollen from the flower buds; (4) homogeneous material that is free of anther tissue can be observed and collected for cellular and molecular studies (Touraev et al., 1996a; Hosp et al., 2007). Reliable protocols for the production of haploid embryos through isolated microspore culture have been set up in rapeseed, tobacco, and barley (Maluszynski et al.,

2003), which have contributed to their development as model systems. However, as in other species, the efficiency of microspore embryogenesis depends on endogenous and exogenous factors, such as genotype, donor plant conditions, developmental stage of the pollen, and culture conditions. Here I focus on the important parameters of microspore culture in *Brassica* species.

2.1 Donor plants: pretreatments and genotype

The temperature at which the donor plants are grown influences the embryogenic response of the cultured microspores/pollen in different species (Lazar et al., 1984; Dunwell et al., 1985). In *Brassica* species, the donor plants are usually grown at higher temperatures (up to 20 ^{o}C) and then moved to lower temperatures (10/5 ^{o}C day/night; preconditioned) just before or immediately after. Cold temperature pretreatment of the donor plants results in a higher frequency of microspore embryogenesis (Ferrie et al., 1995). Microspores from cold treated donor plants are more translucent compare to those without cold treatment (Lo and Pauls, 1992), suggesting that a different physiology is associated with the increased competence for microspore embryogenesis.

Genotypic variation for the ability to produce microspore embryos has been reported in many crops (Powell, 1990). In *Brassica* species, embryogenic response varies among genotypes. To identify genes or loci that are related to microspore embryogenic responsive ability, molecular-genetic analyses, such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) marker analysis, have been performed on different genotypes (Cloutier et al., 1995; Zhang and Takahata, 2001; Zhang et al., 2003). Two loci with multiple genes are associated with embryogenic capacity in *B. napus* (Zhang and Takahata, 2001). However, quantitative trait loci (QTL) studies and fine mapping are needed to find the underlying genes.

2.2 Developmental stage

The developmental stage of pollen is one of key factors for microspore embryogenesis. The developmental stage with embryogenic competence is very narrow (Kyo and Harada, 1986; Pechan and Keller, 1988; Telmer et al., 1992; Binarova et al., 1993). During pollen development in *Brassica* species the uninucleate microspore divides to form a large vegetative cell and a smaller generative cell (Pollen Mitosis I (PMI)). The vegetative cell

arrests in G1 (Žárský et al., 1992), while the generative cell divides once more (PMII) to form two sperm cells. In most species, including *Brassica* spp. microspore embryos are derived from the microspore or vegetative cell of the bicellular pollen grain (Custers et al., 1994; Kasha et al., 2001). Generally only vacuolated microspores prior to PMI or early bicellular pollen just after PMI are responsive for embryo induction. This suggests that a specific stage of the cell cycle is important for efficient embryogenesis from the vegetative cell. However, this developmental window can be widened under specific stress treatments (Touraev et al., 1996a,b). For example, in *Brassica*, higher temperature treatment (41 ^{o}C) can also induce embryogenesis from older stages of bicellular pollen (Binarova et al., 1997). This suggests that a stronger stress treatment is required to bring the vegetative cell back into the cell cycle once it has passed the early binucleate stage.

2.3 Stress treatment

One or more stress treatments are required to induce microspore embryogenesis. These stress treatments include abiotic and/or chemical treatments (Touraev et al., 1997). The widely used abiotic stresses include cold, heat, and carbon starvation. Chemical stresses include colchicine, agents that raise the osmotic pressure and alcohols (Shariatpanahi et al., 2006).

In *B. napus*, the initial culture temperature directs the developmental fate of cultured microspores. Heat treatment at 32 ^{o}C for as little as 8 h is enough to induce sporophytic division, while microspores cultured at 25 ^{o}C or lower temperature follow the gametophytic pathway and develop into trinucleate pollen (Custers et al., 1994). However, as discussed below, long-term culture at 18 ^{o}C can also induce the formation of suspensor-bearing microspore embryos (Prem et al., 2012, see section 5.1).

The initiation of sporophytic division after stress treatment is characterized by the derepression of cell cycle arrest of the vegetative cell and entry into the S phase. At the onset of *B. napus* microspore culture, the microspore nucleus is at G1 to G2 phase of the cell cycle, while the vegetative nucleus of bicellular pollen is arrested at the G1 stage (Binarova et al., 1993). Flow cytometry and bromodeoxyuridine (BrdU) labelling during the heat stress treatment showed that the nucleus of the microspore and the vegetative cell of bicellular pollen rapidly enter S-phase within four hours (Binarova et al., 1993, 1997).

Heat-stress induces the reorganization of the cytoskeleton during microspore embryogenesis. Upon heat treatment, the microtubules reorient and in general, the microspore/ vegetative cell divides symmetrically, rather than asymmetrically, as in pollen development (Hause et al., 1993). The appearance of a pre-prophase band (PPB) of microtubules, which is not found in pollen, can be a marker for embryogenic division. A PPB is observed in *B. napus* microspores after 4-8 h of heat treatment, but not in microspores cultured under non-embryonic conditions or cultured to develop as pollen (Simmonds, 1994; Simmonds and Keller, 1999; Dubas et al., 2011). These results suggest that heat-stress results in the rearrangement of cytoskeleton, and that this rearrangement is associated with sporophytic division. Drugs that influence microtubule and actin polymerization, such as colchicine and cytochalasin D have also been used to induce a low frequency of microspore embryogenesis in *B. napus* (Zaki and Dickinson, 1991; Zhao et al., 1996; Gervais et al., 2000), suggesting that rearrangement of the cytoskeleton is associated with embryo fate switch.

Heat-shock proteins (Hsps) are important components in various stress responses. Hsps are synthesized in *B. napus* microspores after heat stress induction (Pechan et al., 1991; Cordewener et al., 1995; Binarova et al., 1997; Seguí-Simarro et al., 2003). Increased Hsp70 synthesis is detected 8 h after the heat stress treatment, but only at the developmental stage that is competent for embryogenic growth, and not under non-inductive condition (18 ^{o}C) (Cordewener et al., 1995; Binarova et al., 1997). Both Hsp70 and Hsp90 move to the nucleus during microspore embryogenesis induction (Seguí-Simarro et al., 2003). These results suggest that Hsp70 can be used as a marker for stress induced embryogenic switch. However, Hsp70 did not increase in colchicine-induced microspores in the absence of heat stress, suggesting that Hsp activation in microspore culture protects competent microspores/pollen from the harmful effect of heat stress rather than inducing embryogenesis (Zhao et al., 2003).

Different stresses can induce microspore embryogenesis, suggesting that the responses induced by these different stresses converge on a common pathway to activate embryogenesis. Microspores undergo molecular and cellular changes after embryogenic induction, including cytoskeleton rearrangement, cell cycle progression and protein expression (Maraschin et al., 2005; Soriano et al., 2013). Whether or when these events represent convergent points on a common pathway still needs to be determined.

2.5 Conversion to seedlings

Regeneration of microspore embryos into normal plants is an important process for applied DH production and for the generation of homozygous transgenic lines. Unlike zygotic embryos, microspore embryos can germinate into plantlets without having gone through the typical maturation processes that prepare the zygotic embryo for dormancy. Nonetheless, the frequency of plantlet conversion is low in some species. In *B. napus*, the conversion efficiency is around 30% (Belmonte et al., 2006). The conversion efficiency depends on the developmental stage of embryo, the germination medium and other culture conditions.

In *B. napus*, shoot apical meristem (SAM) degeneration has been proposed as the reason for low plant conversion (Yeung et al., 1996). Methods for optimizing culture conditions have involved the inclusion of several compounds in the medium, such as brassinosteroids (Belmonte et al., 2011), abscisic acid (ABA) (Hays et al., 1996), polyethylene glycol (PEG) (Ilić-Grubor et al., 1998b), and buthionine sulfoximine (BSO), which alters the cellular redox status (Stasolla et al., 2008). Transcriptome analysis reveals that BSO stimulates meristem-related and ABA-related genes expression, which related to improved embryo quality (Stasolla et al., 2008). Microspore embryos cultured in the presence of ABA accumulate storage products, as during zygotic embryogenesis, develop well-organized shoot apical meristems (SAMs), and are able to regenerate viable plants at a higher frequency (Wilen et al., 1990; Ramesar-Fortner and Yeung, 2006).

2.6 Chromosome doubling

Plants regenerated from microspore embryos can be haploid, diploid, polyploidy or aneuploid. It is reported that 70–90% of regenerated plants from *B. napus* microspore culture are haploid (Charne and Beversdorf, 1988; Chen and Beversdorf, 1992). Haploid plants are sterile, therefore chromosome doubling is required to regenerate fertile DH plants. Inefficient chromosome doubling is one bottleneck in the production of some DH plants (Germanà, 2011).

Three mechanisms are involved in chromosome duplication, including endoreduplication, nuclear fusion and c-mitosis (Seguí-Simarro and Nuez, 2008). In cereals such as wheat, barley and maize, the efficiency of spontaneous chromosome doubling fusion is quite high (up to 80%) and involves nuclear fusion (Hu and Kasha, 1999; Kasha et al., 2001; Testillano et al., 2004). The efficiency of spontaneous chromosome doubling is quite low in *Brassica* spp. The

antimicrotubule agents colchicine and trifluralin have been used to improve chromosome doubling frequencies in *Brassica* (Zhao and Simmonds, 1995). Colchicine induces microtubule depolymerization, which disrupt chromosome separation during mitosis, resulting in chromosome doubling. Colchicine treatment can be applied at different stages in the DH production process, including isolated microspores/pollen, during the induction stage, microspore embryos (Chen et al., 1994; Möllers et al., 1994; Zhou et al., 2002a; Zhou et al., 2002b), or plantlets (Mathias and Robbelen, 1991). Colchicine treatment at the freshly isolated microspore/pollen stage is more favorable, because it also increases embryo production frequency and reduces the frequency of chimeric plants (Zhou et al., 2002a,b). Understanding of the mechanism of chromosome doubling would help to establish effective methods to produce true homozygous DH plants.

3. Applications of MDE

In vitro microspores culture is the most exploited DH technique and has been broadly applied in breeding programmes and genetic studies, because of its simplicity and higher efficiency for producing homozygous lines in a short period compared to other conventional DH production systems, such as *in vitro* anther culture and gynogenesis (embryo production from the female gametophyte) (Forster et al., 2007).

Homozygous DH plants derived from microspore embryos provide good material for hybrid cultivar production. F1 hybrids can exhibit hybrid vigour (heterosis) and increased yield compared to their parents. F1 hybrids are produced by crossing two homozygous parental lines. In traditional breeding programmes, it takes many generations of selfings to obtain homozygous plants. However, homozygous DHs can be obtained in one generation, and therefore greatly speed up the breeding process. Most of the currently grown *B. napus* varieties are derived from DH technology from microspore culture (Dunwell, 2010; Ferrie and Möllers, 2011). Homozygous DH plants derived from microspore embryos are also used to simplify genome sequencing.

Microspores and microspore embryos are ideal targets for mutagenesis, because microspores are single-celled and because large scale mutagenesis can be performed on thousands of potential plants (donor microspores) at one time. Combine with chromosome duplication, it is possible to produce mutated homozygous plants without chimeras. In *Brassica*, modification of the fatty acid composition is an important objective of breeding

(Friedt and Lühs, 1998). Microspore mutagenesis protocols using ethylmethane sulfonate (EMS) were developed for *B. napus*, *B. juncea*, *B. rapa*, *B. nigra* and *B. carinata*. The fatty acid profiles were evaluated among the DH lines (Barro et al., 2001; Ferrie et al., 2008). Lines exhibiting changes in the level of glucosinolate and erucic acids in *B. carinata* in *B. napus* have been selected from microspores exposed to ultraviolet light (UV) (Barro et al., 2002; McClinchey and Kott, 2008).

Microspore and microspore embryos are ideal targets for genetic modification by transformation. For transformation, it is possible to produce homozygous lines from the primary transformants with the gene of interest. Many transformation techniques have been used to introduce interesting genes into microspores such as microinjection, electroporation, particle bombardment and *Agrobacterium tumefaciens*-mediated transformation (Touraev et al., 2001). The Luciferase gene was introduced into microspores by particle bombardment technique that resulted in fertile and stable transgenic DH plants in *B. napus* (Fukuoka et al., 1998).

Microspore embryogenesis is a good way to produce DH plants from gametes in reverse breeding. The aim of reverse breeding is to genetically fix heterozygous lines with desired traits. Reverse breeding comprises two steps: suppression of crossover recombination during meiosis and production of DH plants from the resulting gametophytes (Dirks et al., 2009). The resulting lines can then be screened for the trait of interest and propagated immortally i.e. without genetic segregation.

Microspore embryogenesis is widely applied in plant breeding. Although reliable microspore embryogenesis protocols have been set up for many species, many important agricultural and commercial plants are still recalcitrant. The low efficiency of embryo induction, plantlet conversion and chromosome doubling are major bottlenecks in DH production. Detailed knowledge on the cellular, biochemical and molecular mechanisms that control the microspore embryogenesis is required to improve existing protocols and to develop species/genotype-independent DH production methods.

4. MDE as a model system to answer fundamental questions in plant biology

Besides its utility for plant breeding and genetics, the microspore embryogenesis system also provides a novel system for understanding plant cell totipotency (Reynolds, 1997; Soriano et al., 2013) and for fundamental studies on other aspects of plant embryogenesis.

Unlike zygotic embryos, microspore embryos are not surrounded by the maternal seed coat or endosperm. This makes it possible to efficiently collect large amounts of staged embryos for subsequent molecular and biochemical analyses, and to directly chemically interrogate different processes. In *B. napus*, this system has been used to identify genes expressed during early embryo development (Joosen et al., 2007; Malik et al., 2007), and to determine the role of the suspensor in embryo development (Supena et al., 2008), the role of regular cell division and polar auxin transport in embryo patterning (Soriano et al., 2014/Chapter 5), the role of histone acetylation in cell fate specification and embryo patterning (Li et al., 2014/Chapter 3), and the regulation of storage product accumulation and hormonal changes during the maturation phase of embryo development (Taylor et al., 1990; Pomeroy et al., 1991; Hays et al., 2001). In barley, anther-derived embryos have been used for studies on storage protein synthesis (Aalen et al., 1994). In tobacco and *B. napus*, genes initially identified in microspore embryo cultures were shown to play important roles during plant development (Boutilier et al., 2002; Hosp et al., 2014). These studies show that despite the morphological and developmental differences between microspore and zygotic embryos, microspore embryos can serve as surrogates for understanding zygotic embryo development.

In this thesis, we used the *B. napus* microspore embryo system to study the role of two processes that are known to have key roles in zygotic embryo development, auxin signaling and chromatin modification. The initial goal of these studies was to understand the specific role of these processes in the establishment and growth of embryogenic microspores/pollen, however, these studies also generated new knowledge and concepts that serve as a framework for studying these processes in zygotic embryo development. Below, I provide an overview on the regulation of zygotic embryo development in relation to auxin signaling and chromatin-level changes in gene expression, and place this information in the context of other studies on *in vitro* embryogenesis.

5. Pattern formation and auxin signaling during zygotic and *in vitro* embryo development

Embryo development has been extensively studied in the model plant Arabidopsis, in part due to the general characteristics that have made this plant a good model system, but also due to the highly regular and predictable division planes of Arabidopsis embryos. *B. napus* zygotic embryos undergo the same regular divisions as Arabidopsis embryos (Tykarska, 1976;

Yeung et al., 1996). Studies on Arabidopsis zygotic embryogenesis therefore provide a solid framework for understanding *B. napus* microspore embryo development.

5.1 Pattern formation during zygotic embryo development

Arabidopsis embryogenesis starts with the one-celled zygote, which results from the fusion of the egg and sperm nuclei after fertilization. The zygote divides asymmetrically to form a small apical cell and a large basal cell, each with distinct developmental fates. The apical cell undergoes two longitudinally divisions and one transverse division to form the eight-cell embryo proper. Periclinal division of these cells results in the formation of the protoderm. The protoderm divides anticlinally to give rise to the epidermis, while the inner cells give rise to the procambium and the ground tissue. The basal cell divides transversely to form the suspensor. The uppermost cell of the suspensor, the hypophysis, forms the root meristem, while the other cells of the suspensor degenerate later in embryo development (Marsden and Meinke, 1985). An initial globular stage of embryo development is followed by the transition to bilateral symmetry and establishment of the apical and basal axis, characterized by establishment of the cotyledon primordia, the provasculature and the shoot and root meristems. The suspensor is a transitory structure that plays an important role in embryo development, as it supports the embryo proper by providing nutrients and growth regulators (Kawashima and Goldberg, 2010).

Arabidopsis is now the most popular model for studies on plant embryogenesis. However, not all flowering plants show the same regular pattern of cell division during zygotic embryo development as Arabidopsis. The first division of the apical cell can be transversal or longitudinal, depending on the species (Mordhorst et al., 1997). For example, in carrot the apical cell divides transversally two times rather than longitudinally (Lackie and Yeung, 1996). Likewise, many variations in suspensor structure can be observed in different species. For example, the suspensor of legumes varies in cell number and arrangement (Yeung and Meinke, 1993; Kawashima and Goldberg, 2010). *Phaseolus coccineus* (scarlet runner bean) has a massive suspensor with a hundred cells arranged in multiple columns (Yeung and Sussex, 1979; Weterings et al., 2001), while *Glycine max* (soybean) has a small suspensor with only a few randomly arranged cells (Chamberlin et al., 1994). These observations indicate that plant embryogenesis is characterized by a high level of plasticity.

Mutant analysis in Arabidopsis suggests that the asymmetric division that generates the initial apical-basal pattern of the embryo is important for subsequent cell fate establishment and morphogenesis. Mutants that fail to establish the correct division plane show subsequent defects in embryo organization or even developmental arrest (Wendrich and Weijers, 2013). For example, mutations in GNOM/EMB30, an ARF-GTPase guanine nucleotide exchange factor (GEF), result in two approximately equally-sized cells after zygote division. Subsequent aberrant division planes in the apical cell lead to a variety of phenotypes, from seedlings that lack a root and have a short hypocotyl and short, fused cotyledons, to amorphous globular structures (Mayer et al., 1993; Shevell et al., 1994; Busch et al., 1996). The WUSCHEL-RELATED HOMEOBOX (WOX) transcription factors WOX2, WOX8 and WOX9 are expressed in the zygote but become restricted to the apical (WOX2) and basal (WOX8, 9) cells after the zygote divides. Mutations in *WOX8* and *WOX9* produce embryos with defects in both basal and apical cell lineages that may arrest later in their development (Breuninger et al., 2008). *WOX8* and *WOX9* regulate expression of *WOX2*, a master regulator of apical cell fate specification and division pattern. *WOX2* expression in the embryo proper rescues apical defects in *wox8 wox9* mutants. The zinc-finger transcription factor WRKY2 ensures the re-establishment of polarity after fertilization through the activation of *WOX8* and *WOX9* expression in the zygote (Ueda et al., 2011). In *wrky2* mutants the zygote fails to polarize and divides symmetrically. Later, *wrky2* embryos fail to develop a distinguishable root pole and the uppermost suspensor cells shows apical characteristics. However, *wrky2* embryos begin to morphologically recover at the heart stage and eventually regain *WOX8*/*WOX9* expression and develop a functional root pole, suggesting that additional routes converge with the WKRY pathway to direct the organization of the basal embryo domain (Ueda et al., 2011).

The defects in the first zygotic division produced by mutations in the *WRKY2*, *WOX* or *GNOM* genes do not interfere with the initial formation of the extra embryonic suspensor. Rather, the development of the basal cell and the formation of the suspensor is regulated by *SHORT SUSPENSOR* (*SSP*), *YODA* (*YDA*), *MITOGEN ACTIVATED KINASE3* (*MPK3*), *MPK 6* and *GROUNDED* (*GRD*) (Lukowitz et al., 2004; Bayer et al., 2009). In loss-of-function mutants of these genes, the elongation and first division of the zygote is disrupted and a suspensor is often completely absent. The *yda* loss-of-function mutant has a short suspensor with longitudinal division in the basal cell lineage, a shorter hypocotyl and root meristem defects,

while a gain-of-function YDA mutant causes exaggerated suspensor growth, but inhibits the development of the proembryo (Lukowitz et al., 2004).

Signaling between the embryo proper and the suspensor is also important for maintaining suspensor identity, as Arabidopsis mutants in which growth of the embryo proper is compromised often show embryo development from the suspensor (Yeung and Meinke, 1993). For example, mutants in the *SUSPENSOR* (*SUS*), *TWIN*, and *RASPBERRY* loci cause irregular divisions in the embryo proper, which are followed by ectopic development of an embryo proper in the suspensor (Schwartz et al., 1994; Vernon and Meinke, 1994; Yadegari et al., 1994; Zhang and Somerville, 1997). These data suggest that the suspensor is a totipotent structure and that signaling from the embryo proper is required to suppress this totipotency during zygotic embryo development.

5.2. Pattern formation in in vitro cultured embryos

Unlike zygotic embryos, *in vitro*-cultured embryos show a less regular pattern of cell division, even in species such as Arabidopsis and *Brassica napus*, where zygotic embryos show highly regular divisions. In *B. napus* microspore embryogenesis, the first division is usually symmetric and can be distinguished from the asymmetric pollen division (Zaki and Dickinson, 1991). Two major pathways leading to microspore embryo development have been observed, with and without a suspensor, which are controlled by the degree and duration of the heat stress treatment and the genotype. With milder heat stress in *B. Topas* DH4079, large amount of embryos with suspensor-like structures are observed, which mimic zygotic embryos in terms of a regularly patterned embryo proper and suspensor (Joosen et al., 2007; Supena et al., 2008). The cell division pattern in suspensorless embryos is initially highly irregular compared to zygotic embryos (Tykarska, 1976; Yeung et al., 1996). Division patterns are only evident in suspensorless embryos once the surrounding pollen wall (exine) bursts (Hause et al., 1994; Ilić-Grubor et al., 1998a; Prem et al., 2012; Soriano et al., 2014/Chapter 5). These observations indicate that regular division patterns and the presence of a suspensor are not prerequisites for functional embryo formation in microspore culture.

Similar observations have been made during somatic embryo culture, where embryos are derived from vegetative cells rather than gametophytic cells. Generally, exogenous plant growth regulators such as auxin and cytokinin are essential for somatic embryo induction, especially the synthetic auxin 2,4-D. Somatic embryos either directly develop from one cell

or two cells of the explant or indirectly from a group of less organized (callus-like) cells. These embryos often lack a suspensor (Mordhorst et al., 1997; Bassuner et al., 2007) and the initial embryogenic cell division can be irregular and depends on the species, the explant and tissue culture conditions (Mordhorst et al., 1997; Suhasini et al., 1997). In Arabidopsis, somatic embryos can be directly induced from immature zygotic embryos on solid medium in the presence of 2,4-D (Gaj, 2001; Bassuner et al., 2007; Kurczyńska et al., 2007). In this system, somatic embryos origin from single or multiple protodermal cells and subprotodermal cells in the cotyledon followed by both periclinal and anticlinal divisions, then develop through similar embryo developmental stages as zygotic embryos, from globular, heart-shaped, torpedo to cotyledonary, but no suspensor formation is observed (Bassuner et al., 2007; Kurczyńska et al., 2007).

In conclusion, regardless of the origin of the embryo, either from a vegetative cell or gametophytic cell, a highly regular cell division pattern and the presence of a suspensor are not essential for the establishment of embryo fate and embryo patterning *in vitro*. The flexibility in the initial morphogenic program of embryogenesis, either *in vitro* or *in planta* raises the question as to the role of these highly regulated divisions in early embryo growth in species such as Arabidopsis and *Brassica*.

5.3 Role of auxin in zygotic embryo development

Auxin plays a critical role in regulating plant development and growth at different stages of the plant life cycle, including embryogenesis. The cell-specific effects of auxin are achieved by the combined processes of auxin perception, auxin response, auxin distribution and auxin homeostasis.

Auxin regulates developmental processes through directly binding to an auxin receptor. Three types of auxin receptors have been identified in Arabidopsis comprising: six TRANSPORT INHIBITOR RESISTANT1/AUXIN F-BOX (TIR1/AFB) proteins, an S-PHASE KINASE-ASSOCIATED PROTEIN 2a (SKP2a), and AUXIN BINDING PROTEIN 1 (ABP1) (Teale et al., 2006; Peer, 2013). TIR1/AFB and SKP2a are the F-box subunits of the SKP1-CULLIN1-F-BOX (SCF) ubiquitin ligase complex ($SCF^{TIR1/AFB}$ and SCF^{SKP2}). Auxin directly binds to TIR1 and stabilizes the interaction between TIR1 and Aux/IAA transcriptional repressors, promoting Aux/IAA degradation via the ubiquitin pathway. Consequently, Aux/IAA repressors are removed from the promoters of target genes of auxin response factors (ARFs), allowing ARF transcription

factors to regulate target gene transcription (Tan et al., 2007). It had been shown that *tir1 afb2 afb3* and *tir1 afb1 afb2 afb3* mutant embryos lack a root and hypocotyl and only have a single cotyledon, phenotypes that resemble those of the *bdl*/*iaa12* or mp/*arf5* mutants (Dharmasiri et al., 2005). Auxin directly binds to SKP2a and induces the degradation of the DPb transcriptional repressor via the ubiquitin pathway, releasing the E2Fc transcription factor to allow cell cycle progression (Jurado et al., 2008; Jurado et al., 2010). ABP1 directly binds to auxin and localizes at the cell periphery (Woo et al., 2002), and is also involved in auxin signaling through a Rho-like GTPase (ROP) pathway to regulate PIN endocytosis (Chen et al., 2012). Embryos from *abp1* mutants arrest at an early stage due to cell elongation and division problems (Chen et al., 2001).

Auxin regulates developmental processes through control of gene transcription. Genes that rapidly respond to auxin contain a short conserved DNA sequence, TGTCTC, referred to as the auxin response element (AuxREs) in their promoters (Ulmasov et al., 1997b). ARFs directly bind to AuxREs to regulate auxin responsive gene transcription (Ulmasov et al., 1997a). ARFs either act as transcriptional activators or repressors (Ulmasov et al., 1999). Aux/IAAs act as transcriptional repressors by binding to ARF transcription factors to repress auxin responsive gene transcription (Ulmasov et al., 1997b; Remington et al., 2004). At low auxin concentrations, Aux/IAAs bind to ARFs and repress auxin responsive gene expression. At high concentrations, auxin stimulates Aux/IAA protein degradation through SCF^{TIR1}ubiquitin ligase complexes and releases the repressive effect of Aux/IAAs on ARF-mediated transcription (Gray et al., 2001; Tiwari et al., 2001). There are 23 ARFs and 29 Aux/IAAs in Arabidopsis that can interact with each other to form heterodimers or homodimers (Kim et al., 1997; Ulmasov et al., 1997a; Weijers et al., 2006). The different combinations of ARFs and Aux/IAAs create a large number of potential protein-protein interactions to temporally and spatially regulate the auxin response.

ARF and Aux/IAA proteins play major roles in regulating auxin response during plant embryogenesis. A temporal and spatial cellular expression map of the complete *ARF* gene family and part of the Aux/IAA gene family during Arabidopsis embryogenesis was developed (Rademacher et al., 2011, 2012). Mutant phenotypes are observed in either gain-of-function Aux/IAA mutants or loss-of-function ARF mutants. For example, the *mp/arf5* loss-of-function mutant lacks a root meristem and has cup-shaped cotyledons (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998), similar to the phenotype of the gain-of-function

(stabilized/auxin insensitive) *bdl/iaa12* mutant (Hamann et al., 1999; Hamann et al., 2002). BDL/IAA12 and MP/ARF5 can form heterodimers and the rootless defects in stabilized *bdl* mutants can be rescued by overexpression of *MP/ARF5*, which indicates an antagonistic relationship between *BDL/IAA12* and *MP/ARF5* in regulating embryo development (Hardtke et al., 2004).

Auxin distribution plays an important role in the initiation of embryogenesis and the establishment of apical-basal axis and pattern formation. The gradient of auxin distribution is achieved by auxin polar transport, which is mediated by a group of efflux and influx facilitators PIN proteins and AUX1 proteins, respectively (Gälweiler et al., 1998; Kleine-Vehn et al., 2006; Wiśniewska et al., 2006). An auxin maximum can be observed by expression of the artificial auxin responsive element *DR5* (Ulmasov et al., 1997b; Sabatini et al., 1999). The zygote divides asymmetrically to produce an apical cell that develops into the embryo proper and a basal cell that divides to form the hypohysis and suspensor. *DR5* is already expressed in this apical cell after division of the zygote. *DR5* remains expressed in the embryo proper until the 32-cell embryo stage. PIN1 is expressed in all cells of the embryo proper and does not initially display any polarity, whereas PIN7 localizes to the apical membrane of the suspensor. The correlation between PIN7 localization and auxin maximum indicates that PIN7 transports auxin upward through the suspensor to the proembryo. From the 32-cells embryo stage onwards PIN1 directs auxin apically to the tips of the cotyledon primordia via the epidermis and basally toward the root via the provascular cells (Friml et al., 2003). At the same time PIN7 localization reverses in the suspensor and PIN4 accumulates in the uppermost suspensor cell, the hypophysis (Friml et al., 2003). The net result is that auxin accumulates in the hypophysis and tips of the cotyledon primordia, and can be indirectly visualized by *DR5* expression in these regions.

The pattern of auxin distribution directed by PIN1 and PIN7 mediates embryo patterning formation and PINs act redundantly in this process. Only mild basal defects are observed in *pin* single mutants at the early embryo stage. Notably, the *pin7* mutant fails to establish an auxin maximum in the embryo proper and shows cell division defects in the basal region of the embryo proper (Friml et al., 2003). More penetrant and severe embryo phenotypes are observed in triple or quadruple *pin* mutants. For example, *pin1 pin3 pin4 pin7* and *pin2 pin3 pin4 pin7* shows disturbed cell division patterns in the basal region of the embryo proper at early embryo stage and displays apical-basal embryo defects including lack of a root

meristem and fused cotyledon or pin-like apical stem at a late embryo stage (Friml et al., 2003). Blilou and coworkers also show misexpression of apical and basal domain markers (Blilou et al., 2005). This data suggests that PIN-mediated auxin distribution is essential for the establishment of apical-basal cell fates during zygotic embryogenesis.

The gradient of auxin distribution not only affects apical-basal axis patterning formation but also affects bilateral symmetry and meristem establishment. In dicotyledonous plants such as Arabidopsis, the two cotyledon primordia are initiated at the late globular stage, and these develop into two separate cotyledons. A local auxin gradient directed by PIN1 redistribution of auxin from the basal to the apical pole promotes cotyledon initiation, and is marked by *DR5* maxima at the tips of the primordia and cotyledons (Benková et al., 2003).

A Knotted-like homeodomain transcription factor SHOOT MERISTEMLESS (STM) and a group of NAC domain transcription factors, CUP-SHAPED COTYLEDON1 (CUC1), CUC2 and CUC3, are essential for shoot apical meristem formation and also involved in repressing the outgrowth of cotyledon boundary. Both single *stm* mutants and double *cuc1 cuc2* mutants lack a shoot apical meristem and display fused cotyledons (Barton and Poethig, 1993; Aida et al., 1997; Long and Barton, 1998; Aida et al., 1999; Vroemen et al., 2003; Hibara et al., 2006).

PIN1 and the protein kinase PINOID (PID), which is a serine-threonine kinase that controls PIN proteins localization by phosphorylating PINs (Friml et al., 2004; Michniewicz et al., 2007), are involved in cotyledon development (Aida et al., 2002; Furutani et al., 2004). *pid* and *pin1* single mutants and *pin1 pid* double mutants have a functional SAM, indicating that PIN1 and PID are not essential for SAM formation (Aida et al., 2002; Furutani et al., 2004). However, both *pid* and *pin1* mutants display cotyledon defects with decreased or increased cotyledon number, size and position. *pid* mutants mostly have three separated cotyledons (Bennett et al., 1995; Treml et al., 2005), while *pin1* mutants have fused cotyledons at the basal region (Bennett et al., 1995; Furutani et al., 2004). *pin1 pid* double mutants completely lack cotyledons and show ectopic expression of *CUC* boundary genes in the cotyledon primordia, which can be rescued by eliminating *CUC* gene expression (Furutani et al., 2004). These results suggest that PIN1 and PID promote cotyledon development through repression of *CUC* gene expression (Aida et al., 2002; Furutani et al., 2004).

The *cuc* mutant phenotype is also observed in Arabidopsis and *Brassica* zygotic embryos and microspore embryos treated with Polar Auxin Transport (PAT) inhibitors (Liu et al., 1993; Hadfi et al., 1998; Ramesar-Fortner and Yeung, 2001; Friml et al., 2003), which confirms that

the graded distribution of auxin is essential for the establishment of bilateral symmetry during embryogenesis.

Auxin homeostasis is determined by the combination of cell-specific auxin biosynthesis, degradation and conjugation. Genes involved in auxin biosynthesis play an important role in embryo development. The YUCCA (YUC) family of flavin monooxygenases are key enzymes in tryptophan-dependent auxin biosynthesis (Zhao, 2010). Due to redundancy among YUCCA family members, single *yuc* mutant do not show developmental defects (Cheng et al., 2006). The *yuc1 yuc4 yuc10 yuc11* quadruple mutant displays embryo defects including lack of a hypocotyl and root meristem, similar to the *mp* mutants. Combination of *yuc* mutants with auxin efflux mutants (*yuc1 yuc4 pin1*) or auxin influx mutants in (*yuc1 yuc4 yuc6 aux1*) or *yuc1 yuc4* mutants treated with auxin polar transport inhibitors display stronger apical defects (with pin-like inflorescences) than *yuc1 yuc4* and *pin1* mutants alone, indicating that auxin biosynthesis genes and polar transport genes synergistically control plant development (Cheng et al., 2007).

5.4 Role of auxin in in vitro embryo development

Microspore embryogenesis in model systems such as *Brassica*, tobacco, barley and wheat, does not require addition of plant growth regulators, such as auxins or cytokinins, to induce embryogenic cell divisions, and as a result most embryos develop directly from the microspore or pollen without an intervening callus phase. The absence of exogenous growth regulators in the medium also makes it easier to study the role of endogenous hormone biosynthesis and signaling in embryo cell fate establishment and morphogenesis.

Application of the anti-auxin PCIB enhances embryo induction and haploid plantlet regeneration in *B. rapa*, *B. napus* and *B. juncea* microspore culture (Agarwal et al., 2006; Zhang et al., 2011; Ahmadi et al., 2012). However, PCIB also disturbs embryo patterning, resulting in embryos that lack cotyledons or have fused cotyledons, similar to the effects of the auxin polar transport inhibitor NPA (Liu et al., 1993; Ramesar-Fortner and Yeung, 2001; Agarwal et al., 2006). Recently, Dubas et al (2013, 2014) showed that endogenous auxin and abscisic acid increased during *B. napus* microspore embryo induction. Using the *DR5*, *PIN7* and *GRP* markers, we showed that apical-basal polarity is established at the globular stage in suspensorless embryos, and after a few cell divisions in suspensor-bearing embryos (Soriano et al., 2014/Chapter 5). Both events coincide with a release of the embryo from the

surrounding exine. We also showed that embryo initiation and polarization in suspensorless embryos does not depend on PAT. PAT was required, however, for bilateral patterning and the formation of the meristems. In contrast to suspensorless embryos, PAT is required for the formation of the embryo proper, as in zygotic embryos.

Auxin also plays an important role in somatic embryogenesis. The majority of mechanistic data on somatic embryogenesis has been obtained using Arabidopsis and several conifer model systems. Arabidopsis somatic embryogenesis can be induced from different tissues using the synthetic auxin 2,4-D. Su et al. (2009) examined secondary somatic embryo formation from embryogenic callus derived from 2,4-D-treated immature embryos. Somatic embryos are induced from the callus after removal of 2,4-D from the culture medium. In this system, alternating areas of high and low auxin, as visualized by *DR5* expression, are set up in the edge regions of the callus. The areas of low auxin, characterized by expression of the shoot apical meristem regulator *WUSCHEL (WUS)*, begin to accumulate auxin, possibly through PIN1-mediated transport, which is followed by somatic embryo growth. Downregulation of *WUS* or *PIN1* expression decreases somatic embryo production (Su et al., 2009). These results suggest that PIN-mediated auxin gradients and shoot-like stem cells are required for somatic embryogenesis.

Gaj et al. (2006) observed that a number of auxin response mutants showed either decreased number of embryos per explant (*axr4-2* and *axr1-3 axr4-2*) or both a reduced number of embryos and responding explants (*axr4-1,* and *aux1-7 axr4-2*). The Arabidopsis auxin response gene *AUXIN RESISTANT4* (*AXR4*) regulates the localization of auxin influx facilitator AUX1 and is involved in PAT (Dharmasiri et al., 2006), while *AXR1* encodes a protein related to ubiquitin-activating enzyme E1 (Leyser et al., 1993). This data suggests that both auxin response and polar auxin transport are required for somatic embryogenesis.

The role of auxin in somatic embryo induction in conifers is not known. At later stages, PAT is important for radial patterning in conifer embryos. Blocking polar auxin transport with NPA increases the endogenous IAA content and disturbs embryo patterning formation in Norway spruce somatic embryo culture, leading to apical and basal aberrations, including fused or aborted cotyledons and abnormal SAM development and a split basal region (Larsson et al., 2008).

Exogenous 2,4-D induces somatic embryogenesis via expression of auxin biosynthesis and response genes. Both *LEAFY COTYLEDON2* (*LEC2*) and *YUC* genes are required for somatic

embryogenesis induction in Arabidopsis (Gaj et al., 2005; Bai et al., 2013). Overexpression of the LEC2 transcription factor induces somatic embryogenesis from Arabidopsis seedlings in the absence of exogenous auxin. *LEC2* rapidly induces *DR5:GUS* accumulation and increases endogenous IAA content by increasing *YUC* gene expression (Wójcikowska et al., 2013). LEC2 directly binds *YUC4* (Stone et al., 2008) and *IAA30* (Braybrook et al., 2006). These results also suggest that LEC2 regulate somatic embryogenesis via the auxin pathway.

Auxin plays an important role in embryo induction and embryo patterning both during zygotic embryogenesis and *in vitro* embryogenesis, and both processes are mediated by auxin signaling components, PAT and auxin homeostasis.

6. Role of epigenetic processes in zygotic and *in vitro* embryo development

Differential gene transcription by complexes of transcription factors is another mechanism to produce diverse, cell-specific developmental outcomes. Differential gene expression depends in part on the presence of specific combinations of transcription factors, but also on how accessible the DNA is to this transcriptional machinery. The accessibility of the transcriptional machinery to DNA is regulated by the chromatin configuration. The basic unit of chromatin is the nucleosome, which contains 147 base pairs of DNA wrapped around a core histone octamer comprising the histones H2A, H2B, H3 and H4. Histone H1 is not incorporated in the nucleosome, but is bound to the linker DNA between nucleosomes. Histone variants such as H2A.Z and H3.3 can also replace the canonical histones in nucleosomes, and there are also additional histone forms of H2A and H3 (Henikoff and Ahmad, 2005). The N-terminal tails of the core histones can be covalently modified by methylation, acetylation, phosphorylation, ubiquitination, ADP ribosylation and sumoylation (Strahl and Allis, 2000), thereby affecting the compactness of the chromatin. Chromatin remodeling factors also affect chromatin packaging by removing or sliding nucleosomes to expose DNA sequences (Narlikar et al., 2002). In general, it is thought that when chromatin becomes less compact, gene transcription is activated; and when chromatin becomes more compact, gene transcription is repressed (Jenuwein and Allis, 2001). Below we discuss the major chromatin modifications that have been shown to play a role in plant embryogenesis, specifically embryo initiation and embryogenic fate repression from seedlings (Millar and Grunstein, 2006; Deal and Henikoff, 2011).

6.1 Zygotic embryogenesis

Histone acetylation status is maintained by reversible enzymatic reactions involving histone deacetylases (HDACs) and histone acetyl transferases (HAT). HAT and HDAC also regulate non-histone protein modifications (Glozak et al., 2005). HDACs catalyze the removal of acetyl groups from lysine residues in the N-terminal tails of the core histones, while HATs add acetyl groups to these lysine residues (Strahl and Allis, 2000). Addition of acetyl-groups on the lysine residues by HATs neutralizes the positive charge of the lysine and decreases the affinity of the histone for DNA, leading to a more open conformation, making the DNA more accessible to the transcriptional machinery. There are three families of HATs: the GCN5-related N-acetyltransferase (GNAT)/NOZ-YBF2/SAS3-SAS2-TIP60 (GNAT/MYST) family, the CREB-binding protein (CBP) family and the $TAF_{II}250$ family (Pandey et al., 2002). Proteins in the GNAT/MYST family have a HAT domain, which is different from the HAT domains from the CBP family and the $TAF_{II}250$ family. All three subfamilies are found in both eukaryotes and prokaryotes.

HAT complexes play an important role at different stages of plant development, including embryo development (Long, 2006), meristem development (Cohen et al., 2009; Kornet and Scheres, 2009), organogenesis (Bertrand et al., 2003), cell differentiation (Sieberer et al., 2003; Anzola et al., 2010) and gametophyte development (Latrasse et al., 2008). The GCN5 HAT, HAG1 acts as suppressor of TOPLESS, which represses root identity genes in the apical domain of embryo (Long et al., 2006). This suggests that HAG1 is needed to de-repress root gene transcription in the apical domain of the embryo. GCN5, which in yeast is found in the SAGA and ADA complexes, works together with ADA2 to control histone acylation in Arabidopsis (Mao et al., 2006). GCN5 regulates root stem cell maintenance, while ADA2b mediates root cell proliferation, both via the PLETHORA pathway (Kornet and Scheres, 2009). *ADA2b/PROPORZ1* (*PRZ1*) is required for histone acetylation and is involved in Arabidopsis morphogenesis in response to auxin. The *prz1* mutant displays reduced histone H3 and H4 acetylation levels and increased callus formation in the presence of auxin (Sieberer et al., 2003; Anzola et al., 2010). Auxin regulates histone acetylation levels through PRZ1 to control cell proliferation by directly repressing expression of the KRP CDK cell-cycle inhibitors (Anzola et al., 2010). These results suggest that histone acetylation and auxin work together to control cell proliferation and cell differentiation.

In plants, there are three families of HDACs: the RPD3/HDA1 family, named after their yeast counterpart reduced potassium deficiency 3 (Rpd3), the NAD-dependent enzymes of the sirtuin family named after the yeast Silent Information Regulator 2 (Sir2) family (Frye, 2000) and the plant-specific HD2 family (Wu et al., 2000). The RPD3/HDA1 and sirtuin subfamilies are found in both prokaryotes and eukaryotes, while the HD2 family is only present in plants. The plant-specific HD2 family may have gained new functions during evolution (Pandey et al., 2002; Hollender and Liu, 2008).

HDACs can be inhibited chemically by HDAC inhibitors (HDACi), which comprise a group of structurally different chemicals that compete with HDAC substrates (e.g. histone tails) for HDACs binding. Rpd3/Hda1 and plant HD2 HDACs have Zn-dependent deactylase activity. The crystal structure of the Rpd3/Hda1 HDACi trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) showed that these HDACi directly interact with the active zinc site at the catalytic pocket of Rpd3/Hda1 HDACs to inhibit their activity (Finnin et al., 1999). Sirtinol is an inhibitor of the NAD-dependent deacetylases of the Sirtuin family, and structure-activity analysis revealed that a hydroxy-napthaldehyde moiety in this HDACi is essential for its inhibitory activity (Grozinger et al., 2001). A chemical genetics approach using HDACi is widely used to study the function of HDACs in biological processes, both in animals and plants (Hollender and Liu, 2008; Marks, 2010).

HDACs play an important role in plant development (Wu et al., 2000; Tian and Chen, 2001; Xu et al., 2005; Tanaka et al., 2008; Cigliano et al., 2013; Kim et al., 2013). Arabidopsis has 18 HDAC genes including *HDA2-19*, *HDT1-4* and *SRT1-2*. Loss-of-function of HDT1, HDA19 or HDA7 resulted in seed abortion (Wu et al., 2000; Tian and Chen, 2001; Cigliano et al., 2013). Application of the HDACi TSA represses seed germination and postgermination growth, while maintaining embryo-specific gene expression. Somatic embryos are eventually formed after 4 weeks of culture from these arrested seedlings. Genetic analysis has shown that inhibition of HDA6 or HDA6/HDA19 contributes to this phenotype. TSA treatment of *hda6 hda19* double mutant is still required to completely arrest postgermination growth, suggesting that additional redundant HDACs repress embryo identity in seedlings (Tanaka et al., 2008). Derepression of transcription in the *hda19* mutant is associated with increased site-specific histone acetylation (Tian et al., 2005). HDA19 directly targets embryo specific genes such as *LEC1* and *LEC2* and represses them in seedlings. In the *hda19* mutant, the ectopic expression of these genes is associated with the increased level of transcriptional

activation marks such as histone H3 acetylation (H3ac), histone H4 acetylation (H4ac), and histone H3 Lys 4 tri-methylation (H3K4me3) and decreased levels of the transcriptional repression mark H3K27me3. This suggests that HDA19 represses embryo identity by directly regulating these embryo specific genes (Zhou et al., 2013).

HDAC proteins form repressive complexes with other corepressors or with chromatin associated proteins, such as DNA methyltransferase and chromatin remodeling factors, to control developmental process by regulating gene transcription. In Arabidopsis, the Groucho/Tup1 corepressor protein TOPLESS directly interact with AP2 and recruits HDA19 to repress floral organ identity genes such as APETALA3, PISTILLATA and SEPALLATA3 (Causier et al., 2012; Krogan et al., 2012). During embryo development, TPL represses genes such as *STM*, *UNUSUAL FLORAL ORGANS* (*UFO*), *WUSCHEL* (*WUS*) and *KNAT1*, which are required for apical embryo development (Long et al., 2002, 2006). *tpl* and *hda19* mutant embryos show similar defects, including a pin-like cotyledon or lack of cotyledons, and the double *tpl hda19* mutant increases the penetrance of apical defects observed in the *tpl* single mutant. This suggests that TPL and HDAC19 repress the same genes during embryogenesis (Long et al., 2006).

It has also been shown that HDACs can also interact with DNA methyltransferase to regulate transposable element (TE) silencing. In the *hda6* mutant, a group of TE are activated due to increased levels of H3 and H4 acetylation and H3K4 methylation. Protein-protein interaction analysis confirms that HDA6 physically interacts with the DNA methyltransferase MET1 to regulate the methylation level of target genes and maintain their silencing status (Liu et al., 2012). In mammalian cells, HDACs can also be recruited by Retinoblastoma (Rb), a master regulator of cell cycle progression, to the promoters of the E2F/DP family of transcription factors (Magnaghi-Jaulin et al., 1998). In mammalian cells, the Rb-E2F repressive complex also interacts with ATP-dependent chromatin remodelers to repress cell proliferation (Zhang et al., 2000). In plants, the maize RBR directly interacts with the ZmRpd3I HDAC protein to repress transcription (Rossi et al., 2003).

Polycomb (PcG) protein complexes also regulate DNA accessibility and transcription through histone modifications. In plants, PcG complexes are associated with transcriptional repression during developmental fate transitions, such as the transition from embryo to vegetative growth or from vegetative to reproductive growth (Hennig and Derkacheva, 2009). The PcG proteins form two main complexes, Polycomb Repressive Complex 1 and 2

(PRC1 and PRC2). PRC1 catalyses monoubiquitination of histone 2A at Lysine 119 (H2AK119u), contributing to transcriptional repression. H2AK119u increases chromatin compaction and blocks the accessibility of chromatin remodeling factors or the transcriptional machinery (Zhou et al., 2008). PRC2 methylates lysine 27 on the N-tail of histone H3 (H3K27), a chromatin mark related to transcriptional repression (Schuettengruber et al., 2011). The core PRC2 complex is conserved between animals and plants. Four core subunits of PRC2 were first identified in Drosophila: ENHANCER OF ZESTE (E(Z)), EXTRA SEX COMBS (ESC), SUPRESSOR OF ZESTE 12 (Su(z)12) and p55. In Arabidopsis, CURLY LEAF (CLF), SWINGER (SWN) and MEDEA (MEA) are homologues of (E(Z)), EMBRYONIC FLOWER2 (EMF2), VERNALIZATION2 (VRN2) and FERTILIZATION-INDEPENDENT SEED2 (FIS2) are homologues of (SU(Z)12), FERTILIZATION-INDEPENDENTENDOSPERM (FIE) is the homologues of (ESC) and MULTICOPY SUPPRESSOR OF IRA1-5 (MSI1-5) is the homologues of p55 (Hennig and Derkacheva, 2009).

There are at least three PRC2-like complexes in Arabidopsis (EMF, FIS, VRN) that have distinct functions during plant development (Guitton and Berger, 2005). PRC2 plays an important role in gametophyte endosperm and seed development. Mutation of any subunit of the FIS2 complex (MEA/SWN, FIS2, FIE and MSI1) leads to endosperm development in the absence of fertilization and eventual seed abortion (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999; Ohad et al., 1999; Köhler et al., 2003a). Both MEA and FIE directly target the same gene, *PHERES1 (PHE1)*. Up-regulated *PHE1* expression is responsible for the seed abortion phenotype in *mea* and *fie* mutants (Köhler et al., 2003b). A reciprocal RBR-PRC2 regulatory circuit exists in reproductive development. It has been shown that RBR regulates PRC2 and MET1 genes. RBR directly binds to *MET1* on the E2F sites. Maternal PRC2 represses the paternal RBR allele during pollen and seed development (Johnston et al., 2008).

PRC2 also plays an important role in repressing embryo identity during the transition from embryo to vegetative (seedling) growth, as single or multiple mutants of *vrn2 emf2* (Schubert et al., 2005), *clf swn* (Chanvivattana et al., 2004) and *fie* (Bouyer et al., 2011) develop ectopic embryos on seedlings after germination. In the *fie* mutant, H3K27me3 deposition seed regulatory genes (e.g. *LEC2*, *ABI3*, *FUS3*) is abolished genome-wide, leading to ectopic activation of their expression (Makarevich et al., 2006; Bouyer et al., 2011). These

results suggest that these PRC2 work as a complex and target the same genes to repress embryogenic identity in vegetative tissue.

PRC1 is also involved in repressing embryo identity in Arabidopsis during the transition to post-germination growth. The core component of PRC1A is a RING-finger protein that functions as an E3 ubiquitin ligases that catalyses H2AK119 ubiquitination (H2AK119u). There are five PRC1 RING-finger genes in Arabidopsis including two RING1 and three BMI1 proteins. Double or triple RING finger mutants (*bmi1a bmi1b*, *bmi1a bmi1b bmi1c* and *ring1a ring1b*) form somatic embryos and show ectopic expression of embryo-specific genes in seedlings (Bratzel et al., 2010; Chen et al., 2010). The level of H2A monoubiquitination of these embryo-specific target genes is reduced in *atbmi1a atbmi1b atbmi1c* mutants, which suggest that PRC1 represses embryo identity in seedlings by catalysing H2AK119u (Bratzel et al., 2010; Yang et al., 2013). The *clf* mutant enhances the penetrance of somatic embryogenesis and enhances embryo-specific gene expression in the *atring1a atring1b* double mutant seedlings (Chen et al., 2010). BMI1 and RING1 can bind to each other and also bind to CLF, EMF1, which deposits the H3K27me3 mark (Turck et al., 2007; Zhang et al., 2007; Xu and Shen, 2008; Bratzel et al., 2010; Chen et al., 2010). This suggests that PRC1 and PRC2 work as complex and play a crucial role in repression embryo traits in seedlings.

A group of ATP-dependent chromatin-remodeling complexes also functions to repress embryo identity during vegetative growth. ATP-dependent chromatin-remodeling complexes alter the chromatin structure by using the energy of ATP hydrolysis to slide or track nucleosomes along DNA (Whitehouse et al., 1999), thereby change the accessibility of DNA to the transcriptional machinery. The ATP-dependent chromatin-remodeling complexes can be divided into three subfamilies: the SWI/SNF family, the Mi-2/CHD family and the ISWI family (Gentry and Hennig, 2014). *PICKLE* (*PKL*) encodes an ATP-dependent chromatin-remodeling factor in the Mi-2/CHD subfamily. There are three homologs in Arabidopsis, *PICKLE RELATED 1* (*PKR1*), *PKR2* and *PKR3*, which all contain three domains: a chromo domain, a SNF2-related helicase/ATPase domain and a DNA-binding domain (Ogas et al., 1999). The *pkl* mutant displays embryogenic traits in roots. These roots show up-regulated expression of embryo specific genes such as *LEC1* and *LEC2* (Ogas et al., 1999; Dean Rider et al., 2003) and can form somatic embryos (Ogas et al., 1997). *PKL* repression of embryo identity in vegetative tissues is associated with the repressive mark H3k27me3 on embryo-specific genes (Zhang et al., 2008). *pkl clf* mutants display increased embryo-specific gene

expression and increased penetrance of the *pkl* root phenotype (Aichinger et al., 2009), which suggests that both PKL and CLF are required to repress embryo genes expression. Aichinger et al. (2009) suggested that PKL indirectly regulates H3k27me3 on embryo-expressed genes by regulating *EMF2, CLF*, *SWN* expression. In contrast, Zhang et al. (2012) have shown that *pkl* does not exhibit decreased transcript levels of PRC2 components and that PKL is present in the promoter region of *LEC1*, *LEC2* and *FUS3*. The differences between these two studies may be due to the use of the *pkl* mutant (Aichinger et al., 2009) compared to a PKL-complemented *pkl* mutant (Zhang et al., 2012). However, both studies found that PKL both up and down regulates H3K27me3-enriched genes, including seed genes, which suggests that PKL can act both as an activator and a repressor (Aichinger et al., 2009; Zhang et al., 2012).

Other ATP dependent chromatin remodeling factors also play an important role in repressing seed storage genes and embryo-related genes in leaf tissue. Loss-of-function mutants of a SNF2 chromatin-remodeling ATPase BRAHMA (BRM), and a homolog of the yeast SWI3 subunit of in SWI/SNF complex (AtSWI3C), as well as an SNF5 homolog BSH display ectopic expression of embryo-related genes in leaf tissue. Chromatin Immunoprecipitation (ChIP) data indicates that BRM represses these embryo related genes by directly binding to them (Tang et al., 2008). These results suggest that chromatin remodeling plays a crucial role in repressing embryo identity in vegetative tissues.

6.2 In vitro embryogenesis

As outlined above, a large number of chromatin associated proteins regulate the transition from embryo to post-germination growth by repressing expression of key regulators of embryo development. Loss-of-function mutants of these regulators show spontaneous somatic embryogenesis, analogous to hormone or stress-induced somatic embryogenesis of cultured explants. In contrast, very little is known about the role of chromatin regulatory proteins during classical *in vitro* embryo culture. Changes in chromatin organization and modification are often associated with off phenotypes (somaclonal variation) in *in vitro* plant regeneration (Miguel and Marum, 2011), but there are few examples where chromatin level changes have been shown to play a direct role *in vitro* embryo induction or growth process.

The mechanism of epigenetic regulation in *in vitro* embryogenesis has been revealed by applying for chemicals that change epigenetic marks. In barley microspore embryogenesis, the DNA methylation inhibitors 5-azacytidine and ethionine enhance callus and embryo induction (Li et al., 2001). In contrast, in *Coffea canephora*, 5-azacytidine inhibits somatic embryo production by decreasing both DNA methylation and expression of *LEC1* and *BBM1.* ChIP assays show that the H3K27me3 repressive mark is associated with reduced *LEC1* and *BBM1* expression (Nic-Can et al., 2013). These results suggest that DNA methylation regulate somatic embryogenesis. In *Picea abies*, TSA treatment enhances embryogenic competence for somatic embryogenesis by increasing the expression of embryo-related genes such as *LEC1* and *VIVIPAROUS1* (*VP1*) (Uddenberg et al., 2011). In Chapters 3 and 4, we show that HDACi treatment enhances embryogenic cell division and embryo yield in *B. napus* microspore culture (Li et al., 2014).

Chromatin-associated proteins work alone or in a complex, to regulate chromatin dynamics and control gene transcription. Most of these factors appear to restrict totipotency *in planta* and during *in vitro* culture.

7. Scope of the thesis

The objective of this thesis was to improve our understanding of haploid embryogenesis. We focused on two aspects of haploid embryogenesis in *B. napus*, the role of chromatin modification and auxin-related processes in haploid embryo induction and embryo development. We have used several different techniques, e.g.marker analysis, chemical perturbation, genetic analysis and transcriptome analysis, to explore the roles of these processes in embryogenesis. The results of this thesis have provided insight into embryo identity and patterning in tissue culture, with implications for the normal establishment and growth of zygotic embryos. In addition, these fundamental studies have generated new tools in the form of chemical inhibitors that can be used to enhance haploid embryo development in other species (Haploid Embryogenesis, PCT/EP2013/069851).

In **Chapter 2**, we summarize the major concepts that have arisen from many years of cell and molecular studies on microspore embryogenesis and put these in the context of more recent experiments and results obtained from the study of pollen and zygotic embryo development.

In **Chapter 3**, we describe the role of HDAC proteins in repressing totipotency during pollen development, using *Brassica napus* and *Arabidopsis* as model systems. Using a set of embryo-expressed GFP reporters and chemical perturbation of HDACs by TSA, we showed that TSA and heat-stress induced similar cellular changes in development, and likely impinge on the same developmental pathways. Genetic analysis in Arabidopsis showed that HDA17 is one of HDACs involved in repressing pollen totipotency, and likely acts redundantly with additional redundant HDACs. Immunoblot analysis showed that TSA induces increased acetylation of histone of H3 and H4 in microspore culture, suggesting that cell proliferation is induced by changes in chromatin marks, rather than by acetylation of non-histone proteins.

In **Chapter 4**, we evaluate the effect of a group of HDACi with a similar mode of action as TSA on embryo induction and yield in *B. napus* microspore culture. We show that a subset of these HDACis are potent enhancers of microspore embryogenesis. The differential specificity of these various HDACi suggests that they target specific HDA proteins. Although HDACi treatment enhances microspore embryo yield, most of the embryogenic multicellular structures induced by HDACi treatment failed to form differentiated embryos. Therefore we performed a transcriptome analysis to identify developmental differences between well-formed embryos and HDACi-induced embryogenic callus. We show that major regulators of embryo domain specification and patterning are down-regulated in embryogenic callus compared to control embryos. We also show that cytokinin and auxin signaling pathways are miss-expressed in these multicellular structures. In contrast, we also show that treatment with HDACi can have a positive effect on embryo patterning, by improving the quality of embryos obtained from older stages of donor pollen. This positive effect on embryo morphology was associated with improved apical basal patterning and an enhanced auxin response. Our results suggest that inhibition of HDAC activity for as short as 20 hours has an impact on later patterning events, perhaps by securing a better commitment to embryonic fate than heat-stress alone.

In **Chapter 5**, we asked the question how microspores and pollen form patterned embryos in the absence of two key events that influence zygotic embryo patterning, an initial symmetric division and the formation of a suspensor. We describe the spatio-temporal expression of an embryo marker (*GRP*) and auxin markers (*DR5*, *PIN1*, *PIN7*) during microspore embryogenesis. We show that in suspensorless embryos, embryo identity was characterized by a (DR5) auxin response and is established before the first sporophytic

division. Embryo polarity in this system is established later than in zygotic embryos, at the globular stage, when the embryo is released from the surrounding exine. PAT was not required for embryo induction or apical-basal polarity establishment, but was required for cotyledon outgrowth and meristem functionality. Using the same markers, we show that suspensor-bearing embryo development proceeds in the same fashion as zygotic embryo development, even when cell divisions are irregular. PAT was required in this system, for the establishment of the embryo proper from the suspensor.

In **Chapter 6**, the main findings of this thesis are summarized and placed in the broader context of the plant development field. We also discuss topics for future research, as well as the possible applications of our research results with respect to microspore embryogenesis in recalcitrant crops.

References

Aalen, R.B., Opsahl-Ferstad, H.G., Linnestad, C., and Olsen, O.A. (1994). Transcripts encoding an oleosin and a dormancy-related protein are present in both the aleurone layer and the embryo of developing barley (*Hordeum vulgare* L.) seeds. Plant J. **5:** 385-396.

Agarwal, P.K., Agarwal, P., Custers, J.B.M., Liu, C.M., and Bhojwani, S.S. (2006). PCIB an antiauxin enhances microspore embryogenesis in microspore culture of *Brassica juncea*. Plant Cell Tissue and Organ Culture **86:** 201-210.

Ahmadi, B., Alizadeh, K., and da Silva, J.A.T. (2012). Enhanced regeneration of haploid plantlets from microspores of *Brassica napus* L. using bleomycin, PCIB, and phytohormones. Plant Cell Tiss. Org. **109:** 525-533.

Aichinger, E., Villar, C.B., Farrona, S., Reyes, J.C., Hennig, L., and Köhler, C. (2009). CHD3 proteins and polycomb group proteins antagonistically determine cell identity in *Arabidopsis*. PLoS Genet. **5:** e1000605.

Aida, M., Ishida, T., and Tasaka, M. (1999). Shoot apical meristem and cotyledon formation during *Arabidopsis* embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes. Development **126:** 1563-1570.

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., and Tasaka, M. (1997). Genes involved in organ separation in Arabidopsis: an analysis of the *cup-shaped cotyledon* mutant. Plant Cell **9:** 841-857.

Aida, M., Vernoux, T., Furutani, M., Traas, J., and Tasaka, M. (2002). Roles of *PIN-FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo. Development **129:** 3965-3974.

Anzola, J.M., Sieberer, T., Ortbauer, M., Butt, H., Korbei, B., Weinhofer, I., Müllner, A.E., and Luschnig, C. (2010). Putative *Arabidopsis* transcriptional adaptor protein (PROPORZ1) is required to modulate histone acetylation in response to auxin. Proc. Natl. Acad. Sci. USA **107:** 10308-10313.

Bai, B., Su, Y.H., Yuan, J., and Zhang, X.S. (2013). Induction of somatic embryos in *Arabidopsis* requires local *YUCCA* expression mediated by the down-regulation of ethylene biosynthesis. Mol. Plant **6:** 1247-1260.

Barro, F., Fernandez‐Escobar, J., De La Vega, M., and Martin, A. (2001). Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. Plant breeding **120:** 262-264.

Barro, F., Fernández-Escobar, J., De la Vega, M., and Martín, A. (2002). Modification of glucosinolate and erucic acid contents in doubled haploid lines of *Brassica carinata* by UV treatment of isolated microspores. Euphytica **129:** 1-6.

Barton, M.K., and Poethig, R.S. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the *shoot meristemless* mutant. Development **119:** 823-831.

Bassuner, B.M., Lam, R., Lukowitz, W., and Yeung, E.C. (2007). Auxin and root initiation in somatic embryos of *Arabidopsis*. Plant Cell Rep. **26:** 1-11.

Bayer, M., Nawy, T., Giglione, C., Galli, M., Meinnel, T., and Lukowitz, W. (2009). Paternal control of embryonic patterning in *Arabidopsis thaliana*. Science **323:** 1485-1488.

Belmonte, M., Elhiti, M., Ashihara, H., and Stasolla, C. (2011). Brassinolide-improved development of *Brassica napus* microspore-derived embryos is associated with increased activities of purine and pyrimidine salvage pathways. Planta **233:** 95-107.

Belmonte, M.F., Ambrose, S.J., Ross, A.R., Abrams, S.R., and Stasolla, C. (2006). Improved development of microspore‐derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism. Physiol. Plant. **127:** 690-700.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115:** 591-602.

Bennett, S.R., Alvarez, J., Bossinger, G., and Smyth, D.R. (1995). Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. Plant J. **8:** 505-520.

Berleth, T., and Jurgens, G. (1993). The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. Development **118:** 575-587.

Bertrand, C., Bergounioux, C., Domenichini, S., Delarue, M., and Zhou, D.-X. (2003). Arabidopsis histone acetyltransferase AtGCN5 regulates the floral meristem activity through the *WUSCHEL/AGAMOUS* pathway. J. Biol. Chem. **278:** 28246-28251.

Binarova, P., Straatman, K., Hause, B., Hause, G., and Van Lammeren, A. (1993). Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. Theor. Appl. Genet. **87:** 9-16.

Binarova, P., Hause, G., Cenklová, V., Cordewener, J.H., and Campagne, M.L. (1997). A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex. Plant Reprod. **10:** 200-208.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature **433:** 39-44.

Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A.A., and Miki, B.L. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell **14:** 1737-1749.

Bouyer, D., Roudier, F., Heese, M., Andersen, E.D., Gey, D., Nowack, M.K., Goodrich, J., Renou, J.-P., Grini, P.E., and Colot, V. (2011). Polycomb repressive complex 2 controls the embryo-to-seedling phase transition. PLoS Genet. **7:** e1002014.

Bratzel, F., López-Torrejón, G., Koch, M., Del Pozo, J.C., and Calonje, M. (2010). Keeping cell identity in *Arabidopsis* requires PRC1 RING-finger homologs that catalyze H2A monoubiquitination. Curr. Biol. **20:** 1853-1859.

Braybrook, S.A., Stone, S.L., Park, S., Bui, A.Q., Le, B.H., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2006). Genes directly regulated by LEAFY COTYLEDON2 provide insight into the control of embryo maturation and somatic embryogenesis. Proceedings of the National Academy of Sciences of the United States of America **103:** 3468-3473.

Breuninger, H., Rikirsch, E., Hermann, M., Ueda, M., and Laux, T. (2008). Differential expression of *WOX* genes mediates apical-basal axis formation in the *Arabidopsis* embryo. Dev. Cell **14:** 867-876.

Busch, M., Mayer, U., and Jürgens, G. (1996). Molecular analysis of the *Arabidopsis* pattern formation gene *GNOM*: gene structure and intragenic complementation. Mol. Gen. Genet. **250:** 681-691.

Causier, B., Ashworth, M., Guo, W., and Davies, B. (2012). The TOPLESS interactome: a framework for gene repression in Arabidopsis. Plant Physiol. **158:** 423-438.

Chamberlin, M.A., Horner, H.T., and Palmer, R.G. (1994). Early endosperm, embryo, and ovule development in *Glycine max* (L.) Merr. Int. J. Plant Sci.**:** 421-436.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.-H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in *Arabidopsis*. Development **131:** 5263-5276.

Charne, D., and Beversdorf, W. (1988). Improving microspore culture as a rapeseed breeding tool: the use of auxins and cytokinins in an induction medium. Can. J. Bot. **66:** 1671-1675.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **94:** 4223-4228.

Chen, D., Molitor, A., Liu, C., and Shen, W.-H. (2010). The *Arabidopsis* PRC1-like ring-finger proteins are necessary for repression of embryonic traits during vegetative growth. Cell Res. **20:** 1332-1344.

Chen, J.-G., Ullah, H., Young, J.C., Sussman, M.R., and Jones, A.M. (2001). ABP1 is required for organized cell elongation and division in *Arabidopsis* embryogenesis. Genes Dev. **15:** 902-911.

Chen, J., and Beversdorf, W. (1992). Production of spontaneous diploid lines from isolated microspores following cryopreservation in spring rapeseed (*Brassica napus* L.). Plant Breeding **108:** 324-327.

Chen, X., Naramoto, S., Robert, S., Tejos, R., Löfke, C., Lin, D., Yang, Z., and Friml, J. (2012). ABP1 and ROP6 GTPase signaling regulate clathrin-mediated endocytosis in *Arabidopsis* roots. Curr. Biol. **22:** 1326-1332.

Chen, Z., Snyder, S., Fan, Z., and Loh, W. (1994). Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breeding **113:** 217-221.

Cheng, Y., Dai, X., and Zhao, Y. (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev. **20:** 1790-1799.

Cheng, Y., Dai, X., and Zhao, Y. (2007). Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. Plant Cell **19:** 2430-2439.

Cigliano, R.A., Cremona, G., Paparo, R., Termolino, P., Perrella, G., Gutzat, R., Consiglio, M.F., and Conicella, C. (2013). Histone deacetylase AtHDA7 is required for female gametophyte and embryo development in *Arabidopsis*. Plant Physiol. **163:** 431-440.

Cloutier, S., Cappadocia, M., and Landry, B. (1995). Study of microspore-culture responsiveness in oilseed rape (*Brassica napus L.*) by comparative mapping of a F2 population and two microspore-derived populations. Theor. Appl. Genet. **91:** 841-847.

Cohen, R., Schocken, J., Kaldis, A., Vlachonasios, K.E., Hark, A.T., and McCain, E.R. (2009). The histone acetyltransferase GCN5 affects the inflorescence meristem and stamen development in *Arabidopsis*. Planta **230:** 1207-1221.

Cordewener, J.H., Hause, G., Görgen, E., Busink, R., Hause, B., Dons, H.J., Van Lammeren, A.A., Campagne, M.M.V.L., and Pechan, P. (1995). Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta **196:** 747-755.

Custers, J.B.M., Cordewener, J.H.G., Nollen, Y., Dons, H.J.M., and Campagne, M.M.V. (1994). Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep. **13:** 267-271.

Deal, R.B., and Henikoff, S. (2011). Histone variants and modifications in plant gene regulation. Curr. Opin. Plant Biol. **14:** 116-122.

Dean Rider, S., Henderson, J.T., Jerome, R.E., Edenberg, H.J., Romero‐Severson, J., and Ogas, J. (2003). Coordinate repression of regulators of embryonic identity by *PICKLE* during germination in *Arabidopsis*. Plant J. **35:** 33-43.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jürgens, G., and Estelle, M. (2005). Plant development is regulated by a family of auxin receptor F box proteins. Dev. Cell **9:** 109-119.

Dharmasiri, S., Swarup, R., Mockaitis, K., Dharmasiri, N., Singh, S., Kowalchyk, M., Marchant, A., Mills, S., Sandberg, G., and Bennett, M. (2006). AXR4 is required for localization of the auxin influx facilitator AUX1. Science **312:** 1218-1220.

Dirks, R., Van Dun, K., De Snoo, C.B., Van Den Berg, M., Lelivelt, C.L., Voermans, W., Woudenberg, L., De Wit, J.P., Reinink, K., and Schut, J.W. (2009). Reverse breeding: a novel breeding approach based on engineered meiosis. Plant Biotechnol. J. **7:** 837-845.

Dubas, E., Custers, J., Kieft, H., Wędzony, M., and van Lammeren, A.A. (2011). Microtubule configurations and nuclear DNA synthesis during initiation of suspensor-bearing embryos from *Brassica napus* cv. Topas microspores. Plant Cell Rep. **30:** 2105-2116.

Dubas, E., Benkova, E., Janowiak, F., Waligórski, P., Dziurka, M., Krzewska, M., and Żur, I. (2013). Endogenous auxin-and ABA-mediated microspore embryogenesis in *Brassica napus* L. Acta Bio. Cracov. Bot. **55:** 15.

Dubas, E., Moravčíková, J., Libantová, J., Matušíková, I., Benková, E., Żur, I., and Krzewska, M. (2014). The influence of heat stress on auxin distribution in transgenic *B. napus* microspores and microspore-derived embryos. Protoplasma**:** 1-11.

Dunwell, J., Cornish, M., and De Courcel, A. (1985). Influence of genotype, plant growth temperature and anther incubation temperature on microspore embryo production in *Brassica napus ssp. oleifera*. J. Exp. Bot. **36:** 679-689.

Dunwell, J.M. (2010). Haploids in flowering plants: origins and exploitation. Plant Biotechnol. J. **8:** 377-424.

Ferrie, A., Epp, D., and Keller, W. (1995). Evaluation of *Brassica rapa L.* genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Rep. **14:** 580-584.

Ferrie, A., Taylor, D., Mackenzie, S., Rakow, G., Raney, J., and Keller, W. (2008). Microspore mutagenesis of *Brassica* species for fatty acid modifications: a preliminary evaluation. Plant Breeding **127:** 501-506.

Ferrie, A.M., and Möllers, C. (2011). Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss. Org. **104:** 375-386.

Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R., and Pavletich, N.P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature **401:** 188-193.

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

Friedt, W., and Lühs, W. (1998). Recent developments and perspectives of industrial rapeseed breeding. Lipid/Fett **100:** 219-226.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical–basal axis of *Arabidopsis*. Nature **426:** 147-153.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., and Sandberg, G. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306:** 862-865.

Frye, R.A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. **273:** 793-798.

Fukuoka, H., Ogawa, T., Matsuoka, M., Ohkawa, Y., and Yano, H. (1998). Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L.) and the production of fertile transgenic plants. Plant Cell Rep. **17:** 323-328.

Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M., and Aida, M. (2004). *PIN-FORMED1* and *PINOID* regulate boundary formation and cotyledon development in *Arabidopsis* embryogenesis. Development **131:** 5021-5030.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science **282:** 2226-2230.

Gaj, M.D. (2001). Direct somatic embryogenesis as a rapid and efficient system for *in vitro* regeneration of *Arabidopsis thaliana*. Plant Cell Tiss. Org. **64:** 39-46.

Gaj, M.D., Zhang, S., Harada, J.J., and Lemaux, P.G. (2005). Leafy cotyledon genes are essential for induction of somatic embryogenesis of *Arabidopsis*. Planta **222:** 977-988.

Gaj, M.D., Trojanowska, A., Ujczak, A., Mędrek, M., Kozioł, A., and Garbaciak, B. (2006). Hormone-response mutants of *Arabidopsis thaliana* (L.) Heynh. impaired in somatic embryogenesis. Plant Growth Regul. **49:** 183-197.

Gentry, M., and Hennig, L. (2014). Remodelling chromatin to shape development of plants. Exp. Cell Res. **321:** 40-46.

Germanà, M.A. (2011). Anther culture for haploid and doubled haploid production. Plant Cell Tiss. Org. **104:** 283-300.

Gervais, C., Newcomb, W., and Simmonds, D.H. (2000). Rearrangement of the actin filament and microtubule cytoskeleton during induction of microspore embryogenesis in *Brassica napus L.* cv. Topas. Protoplasma **213:** 194-202.

Glozak, M.A., Sengupta, N., Zhang, X., and Seto, E. (2005). Acetylation and deacetylation of non-histone proteins. Gene **363:** 15-23.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001). Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature **414:** 271-276.

Grossniklaus, U., Vielle-Calzada, J.-P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by *MEDEA*, a *polycomb* group gene in *Arabidopsis*. Science **280:** 446-450.

Grozinger, C.M., Chao, E.D., Blackwell, H.E., Moazed, D., and Schreiber, S.L. (2001). Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J. Biol. Chem. **276:** 38837-38843.

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

Guitton, A., and Berger, F. (2005). Control of reproduction by Polycomb Group complexes in animals and plants. Int. J. Dev. Biol. **49:** 707.

Hadfi, K., Speth, V., and Neuhaus, G. (1998). Auxin-induced developmental patterns in *Brassica juncea* embryos. Development **125:** 879-887.

Hamann, T., Mayer, U., and Jurgens, G. (1999). The auxin-insensitive *bodenlos* mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. Development **126:** 1387-1395.

Hamann, T., Benkova, E., Bäurle, I., Kientz, M., and Jürgens, G. (2002). The *Arabidopsis BODENLOS* gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. Genes Dev. **16:** 1610-1615.

Hardtke, C.S., and Berleth, T. (1998). The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. EMBO J. **17:** 1405-1411.

Hardtke, C.S., Ckurshumova, W., Vidaurre, D.P., Singh, S.A., Stamatiou, G., Tiwari, S.B., Hagen, G., Guilfoyle, T.J., and Berleth, T. (2004). Overlapping and non-redundant functions of the *Arabidopsis* auxin response factors *MONOPTEROS* and *NONPHOTOTROPIC HYPOCOTYL 4*. Development **131:** 1089-1100.

Hause, B., Hause, G., Pechan, P., and van Lammeren, A.A.M. (1993). Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol. Int. **17:** 153-168.

Hause, B., Veenendaal, W.v., Hause, G., and Lammeren, A.v. (1994). Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv. Topas. Bot. Acta.

Hays, D., Mandel, R., and Pharis, R. (2001). Hormones in zygotic and microspore embryos of *Brassica napus*. Plant Growth Regul. **35:** 47-58.

Hays, D., Rose, P., Abrams, S., and Moloney, M. (1996). Biological activity of optically pure C-1 altered abscisic acid analogs in *Brassica napus* microspore embryos. J. Plant Growth Regul. **15:** 5-11.

Heberle-Bors, E. (1985). *In vitro* haploid formation from pollen: a critical review. Theor. Appl. Genet. **71:** 361-374.

Henikoff, S., and Ahmad, K. (2005). Assembly of variant histones into chromatin. Annu. Rev. Cell Dev. Biol. **21:** 133-153.

Hennig, L., and Derkacheva, M. (2009). Diversity of Polycomb group complexes in plants: same rules, different players? Trends Genet. **25:** 414-423.

Hibara, K.-i., Karim, M.R., Takada, S., Taoka, K.-i., Furutani, M., Aida, M., and Tasaka, M. (2006). *Arabidopsis CUP-SHAPED COTYLEDON3* regulates postembryonic shoot meristem and organ boundary formation. Plant Cell **18:** 2946-2957.

Hollender, C., and Liu, Z. (2008). Histone deacetylase genes in *Arabidopsis* development. J. Integr. Plant Biol. **50:** 875-885.

Hosp, J., de Faria Maraschin, S., Touraev, A., and Boutilier, K. (2007). Functional genomics of microspore embryogenesis. Euphytica **158:** 275-285.

Hosp, J., Ribarits, A., Retzer, K., Jin, Y., Tashpulatov, A., Resch, T., Friedmann, C., Ankele, E., Voronin, V., and Palme, K. (2014). A tobacco homolog of DCN1 is involved in pollen development and embryogenesis. Plant Cell Rep.**:** 1-16.

Hu, T., and Kasha, K.J. (1999). A cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum* L.) cv. Chris. Genome **42:** 432-441.

Ilić-Grubor, K., Attree, S.M., and Fowke, L.C. (1998a). Comparative morphological study of zygotic and microspore-derived embryos of *Brassica napus* L. as revealed by scanning electron microscopy. Ann. Bot. **82:** 157-165.

Ilić-Grubor, K., Attree, S., and Fowke, L. (1998b). Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. Plant Cell Rep. **17:** 329-333.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science **293:** 1074-1080.

Johnston, A.J., Matveeva, E., Kirioukhova, O., Grossniklaus, U., and Gruissem, W. (2008). A dynamic reciprocal *RBR*-PRC2 regulatory circuit controls *Arabidopsis* gametophyte development. Curr. Biol. **18:** 1680-1686.

Joosen, R., Cordewener, J., Supena, E.D.J., Vorst, O., Lammers, M., Maliepaard, C., Zeilmaker, T., Miki, B., America, T., and Custers, J. (2007). Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol. **144:** 155-172.

Jurado, S., Díaz‐Triviño, S., Abraham, Z., Manzano, C., Gutierrez, C., and Pozo, C.d. (2008). SKP2A, an F‐box protein that regulates cell division, is degraded via the ubiquitin pathway. Plant J. **53:** 828-841.

Jurado, S., Abraham, Z., Manzano, C., López-Torrejón, G., Pacios, L.F., and Del Pozo, J.C. (2010). The Arabidopsis cell cycle F-box protein SKP2A binds to auxin. Plant Cell **22:** 3891-3904.

Köhler, C., Hennig, L., Bouveret, R., Gheyselinck, J., Grossniklaus, U., and Gruissem, W. (2003a). *Arabidopsis* MSI1 is a component of the MEA/FIE *Polycomb* group complex and required for seed development. EMBO J. **22:** 4804-4814.

Köhler, C., Hennig, L., Spillane, C., Pien, S., Gruissem, W., and Grossniklaus, U. (2003b). The *Polycomb*-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene *PHERES1*. Genes Dev. **17:** 1540-1553.

Kasha, K., Hu, T., Oro, R., Simion, E., and Shim, Y. (2001). Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. J. Exp. Bot. **52:** 1227-1238.

Kawashima, T., and Goldberg, R.B. (2010). The suspensor: not just suspending the embryo. Trends Plant Sci. **15:** 23-30.

Kim, J., Harter, K., and Theologis, A. (1997). Protein–protein interactions among the Aux/IAA proteins. Proc. Natl. Acad. Sci. USA **94:** 11786-11791.

Kim, W., Latrasse, D., Servet, C., and Zhou, D.-X. (2013). Arabidopsis histone deacetylase HDA9 regulates flowering time through repression of AGL19. Biochem. Biophys. Res. Commun. **432:** 394-398.

Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., Katz, A., Margossian, L., Harada, J.J., and Goldberg, R.B. (1999). Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **96:** 4186-4191.

Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M., and Friml, J. (2006). Subcellular trafficking of the *Arabidopsis* auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell **18:** 3171-3181.

Kornet, N., and Scheres, B. (2009). Members of the GCN5 histone acetyltransferase complex regulate PLETHORA-mediated root stem cell niche maintenance and transit amplifying cell proliferation in *Arabidopsis*. Plant Cell **21:** 1070-1079.

Krogan, N.T., Hogan, K., and Long, J.A. (2012). APETALA2 negatively regulates multiple floral organ identity genes in *Arabidopsis* by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. Development **139:** 4180-4190.

Kurczyńska, E.U., Gaj, M.D., Ujczak, A., and Mazur, E. (2007). Histological analysis of direct somatic embryogenesis in *Arabidopsis thaliana* (L.) Heynh. Planta **226:** 619-628.

Kyo, M., and Harada, H. (1986). Control of the developmental pathway of tobacco pollen *in vitro*. Planta **168:** 427-432.

Lackie, S., and Yeung, E.C. (1996). Zygotic embryo development in *Daucus carota*. Can. J. Bot. **74:** 990-998.

Larsson, E., Sitbon, F., Ljung, K., and Von Arnold, S. (2008). Inhibited polar auxin transport results in aberrant embryo development in Norway spruce. New Phytol. **177:** 356-366.

Latrasse, D., Benhamed, M., Henry, Y., Domenichini, S., Kim, W., Zhou, D.-X., and Delarue, M. (2008). The MYST histone acetyltransferases are essential for gametophyte development in *Arabidopsis*. BMC Plant Biol. **8:** 121.

Lazar, M., Baenziger, P., and Schaeffer, G. (1984). Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor. Appl. Genet. **68:** 131-134.

Leyser, H.O., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J., and Estelle, M. (1993). *Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1.

Li, H., Soriano, M., Cordewener, J., Muiño, J.M., Riksen, T., Fukuoka, H., Angenent, G.C., and Boutilier, K. (2014). The histone deacetylase inhibitor trichostatin A promotes totipotency in the male gametophyte. Plant Cell**:** tpc. 113.116491.

Li, W.-Z., Song, Z.-H., Guo, B.-T., and Xu, L.-J. (2001). The effects of DNA hypomethylating drugs on androgenesis in barley (*Hordeum vulgare* L.). In VIitro Cell. Dev. - Pl. **37:** 605-608.

Lichter, R. (1982). Induction of haploid plants from isolated pollen of *Brassica napus*. Zeitschrift für Pflanzenphysiologie **105:** 427-434.

Liu, C., Xu, Z., and Chua, N.-H. (1993). Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell **5:** 621-630.

Liu, X., Yu, C.-W., Duan, J., Luo, M., Wang, K., Tian, G., Cui, Y., and Wu, K. (2012). HDA6 directly interacts with DNA methyltransferase MET1 and maintains transposable element silencing in *Arabidopsis*. Plant Physiol. **158:** 119-129.

Lo, K.-H., and Pauls, K.P. (1992). Plant growth environment effects on rapeseed microspore development and culture a flow cytometric study. Plant Physiol. **99:** 468-472.

Long, J.A. (2006). Plant development: new models and approaches bring progress. Development **133:** 4609-4612.

Long, J.A., and Barton, M.K. (1998). The development of apical embryonic pattern in *Arabidopsis*. Development **125:** 3027-3035.

Long, J.A., Ohno, C., Smith, Z.R., and Meyerowitz, E.M. (2006). TOPLESS regulates apical embryonic fate in *Arabidopsis*. Science **312:** 1520-1523.

Long, J.A., Woody, S., Poethig, S., Meyerowitz, E.M., and Barton, M.K. (2002). Transformation of shoots into roots in *Arabidopsis* embryos mutant at the *TOPLESS* locus. Development **129:** 2797-2806.

Lukowitz, W., Roeder, A., Parmenter, D., and Somerville, C. (2004). A MAPKK kinase gene regulates extra-embryonic cell fate in *Arabidopsis*. Cell **116:** 109-119.

Möllers, C., Iqbal, M., and Röbbelen, G. (1994). Efficient production of doubled haploid Brassica napus plants by colchicine treatment of microspores. Euphytica **75:** 95-104.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature **391:** 601-605.

Makarevich, G., Leroy, O., Akinci, U., Schubert, D., Clarenz, O., Goodrich, J., Grossniklaus, U., and Köhler, C. (2006). Different *Polycomb* group complexes regulate common target genes in *Arabidopsis*. EMBO Rep. **7:** 947-952.

Malik, M.R., Wang, F., Dirpaul, J.M., Zhou, N., Polowick, P.L., Ferrie, A.M., and Krochko, J.E. (2007). Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol. **144:** 134-154.

Maluszynski, M., Kasha, K.J., B.P., F., and I., S. (2003). Doubled haploid production in crop plants: a manual. (Dordrecht, The Netherlands: Kluwer Academic Publishers).

Mao, Y., Pavangadkar, K.A., Thomashow, M.F., and Triezenberg, S.J. (2006). Physical and functional interactions of *Arabidopsis* ADA2 transcriptional coactivator proteins with the acetyltransferase GCN5 and with the cold-induced transcription factor CBF1. Biochim. Biophys. Acta - Gene Struct. Expression **1759:** 69-79.

Maraschin, S.F., de Priester, W., Spaink, H.P., and Wang, M. (2005). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. J. Exp. Bot. **56:** 1711-1726.

Marks, P.A. (2010). Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. Biochim. Biophys. Acta - Gene Regul. Mech. **1799:** 717-725.

Marsden, M.P., and Meinke, D.W. (1985). Abnormal development of the suspensor in an embryo-lethal mutant of *Arabidopsis thaliana*. Am. J. Bot.**:** 1801-1812.

Mathias, R., and Robbelen, G. (1991). Effective diploidization of microspore-derived haploids of rape (*Brassica napus* L.) by invitro colchicine treatment. Plant Breeding **106:** 82-84.

Mayer, U., Buttner, G., and Jurgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development **117:** 149-162.

McClinchey, S.L., and Kott, L.S. (2008). Production of mutants with high cold tolerance in spring canola (*Brassica napus*). Euphytica **162:** 51-67.

Michniewicz, M., Zago, M.K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M.G., Ohno, C., Zhang, J., and Huang, F. (2007). Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell **130:** 1044-1056.

Miguel, C., and Marum, L. (2011). An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. J. Exp. Bot. **62:** 3713-3725.

Millar, C.B., and Grunstein, M. (2006). Genome-wide patterns of histone modifications in yeast. Nat. Rev. Mol. Cell Biol. **7:** 657-666.

Mordhorst, A.P., Toonen, M.A., de Vries, S.C., and Meinke, D. (1997). Plant embryogenesis. Crit. Rev. Plant Sci. **16:** 535-576.

Narlikar, G.J., Fan, H.-Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell **108:** 475-487.

Nic-Can, G.I., López-Torres, A., Barredo-Pool, F., Wrobel, K., Loyola-Vargas, V.M., Rojas-Herrera, R., and De-la-Peña, C. (2013). New insights into somatic embryogenesis: *LEAFY COTYLEDON1*, *BABY BOOM1* and *WUSCHEL-RELATED HOMEOBOX4* are epigenetically regulated in *Coffea canephora*. PloS one **8:** e72160.

Nitsch, C. (1974). La culture de pollen isolé sur milieu synthetique. CR Acad. Sci. Paris **278:** 1031-1034.

Ogas, J., Cheng, J.-C., Sung, Z.R., and Somerville, C. (1997). Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana pickle* mutant. Science **277:** 91-94.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **96:** 13839-13844.

Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P., and Fischer, R.L. (1996). A mutation that allows endosperm development without fertilization. Proc. Natl. Acad. Sci. USA **93:** 5319-5324.

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Mutations in *FIE*, a WD polycomb group gene, allow endosperm development without fertilization. Plant Cell **11:** 407-415.

Pandey, R., MuÈller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W., and Jorgensen, R.A. (2002). Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res. **30:** 5036-5055.

Pechan, P., Bartels, D., Brown, D., and Schell, J. (1991). Messenger-RNA and protein changes associated with induction of Brassica microspore embryogenesis. Planta **184:** 161-165.

Pechan, P.M., and Keller, W.A. (1988). Identification of potentially embryogenic microspores in *Brassica napus*. Physiol. Plant. **74:** 377-384.

Peer, W.A. (2013). From perception to attenuation: auxin signalling and responses. Curr. Opin. Plant Biol. **16:** 561-568.

Pomeroy, M., Kramer, J., Hunt, D., and Keller, W. (1991). Fatty acid changes during development of zygotic and microspore‐derived embryos of *Brassica napus*. Physiol. Plant. **81:** 447-454.

Powell, W. (1990). Environmental and genetical aspects of pollen embryogenesis. In Haploids in Crop Improvement I, Y. Bajaj, ed (Berlin: Springer-Verlag), pp. 45-65.

Prem, D., Solís, M.-T., Bárány, I., Rodríguez-Sanz, H., Risueño, M.C., and Testillano, P.S. (2012). A new microspore embryogenesis system under low temperature which mimics zygotic embryogenesis initials, expresses auxin and efficiently regenerates doubled-haploid plants in *Brassica napus*. BMC Plant Biol. **12:** 127.

Rademacher, E.H., Möller, B., Lokerse, A.S., Llavata‐Peris, C.I., van den Berg, W., and Weijers, D. (2011). A cellular expression map of the Arabidopsis *AUXIN RESPONSE FACTOR* gene family. Plant J. **68:** 597-606.

Rademacher, E.H., Lokerse, A.S., Schlereth, A., Llavata-Peris, C.I., Bayer, M., Kientz, M., Freire Rios, A., Borst, J.W., Lukowitz, W., and Jürgens, G. (2012). Different auxin response machineries control distinct cell fates in the early plant embryo. Dev. Cell **22:** 211-222.

Ramesar-Fortner, N.S., and Yeung, E.C. (2001). Tri-iodobenzoic acid affects shoot apical meristem formation and function in zygotic embryos of *Brassica napus* cv. Topas. Can. J. Bot. **79:** 265-273.

Ramesar-Fortner, N.S., and Yeung, E.C. (2006). Physiological influences in the development and function of the shoot apical meristem of microspore-derived embryos of *Brassica napus* ' Topas'. Botany **84:** 371-383.

Remington, D.L., Vision, T.J., Guilfoyle, T.J., and Reed, J.W. (2004). Contrasting modes of diversification in the *Aux/IAA* and *ARF* gene families. Plant Physiol. **135:** 1738-1752.

Reynolds, T.L. (1997). Pollen embryogenesis. Plant Mol. Biol. **33:** 1-10.

Rossi, V., Locatelli, S., Lanzanova, C., Boniotti, M.B., Varotto, S., Pipal, A., Goralik-Schramel, M., Lusser, A., Gatz, C., and Gutierrez, C. (2003). A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription. Plant Mol. Biol. **51:** 401-413.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., and Weisbeek, P. (1999). An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell **99:** 463-472.

Schubert, D., Clarenz, O., and Goodrich, J. (2005). Epigenetic control of plant development by Polycomb-group proteins. Curr. Opin. Plant Biol. **8:** 553-561.

Schuettengruber, B., Martinez, A.-M., Iovino, N., and Cavalli, G. (2011). Trithorax group proteins: switching genes on and keeping them active. Nat. Rev. Mol. Cell Biol. **12:** 799-814.

Schwartz, B.W., Yeung, E.C., and Meinke, D.W. (1994). Disruption of morphogenesis and transformation of the suspensor in abnormal *sus*pensor mutants of *Arabidopsis*. Development **120:** 3235-3245.

Seguí-Simarro, J., and Nuez, F. (2008). Pathways to doubled haploidy: chromosome doubling during androgenesis. Cytogenet. Genome Res. **120:** 358-369.

Seguí-Simarro, J., Testillano, P., and Risueno, M. (2003). Hsp70 and Hsp90 change their expression and subcellular localization after microspore embryogenesis induction in *Brassica napus* L. J. Struct. Bio. **142:** 379-391.

Shariatpanahi, M.E., Bal, U., Heberle‐Bors, E., and Touraev, A. (2006). Stresses applied for the re‐programming of plant microspores towards *in vitro* embryogenesis. Physiol. Plant. **127:** 519-534.

Shevell, D.E., Leu, W.-M., Gillmor, C.S., Xia, G., Feldmann, K.A., and Chua, N.-H. (1994). *EMB30* is essential for normal cell division, cell expansion, and cell adhesion in Arabidopsis and encodes a protein that has similarity to Sec7. Cell **77:** 1051-1062.

Sieberer, T., Hauser, M.-T., Seifert, G.J., and Luschnig, C. (2003). *PROPORZ1*, a putative *Arabidopsis* transcriptional adaptor protein, mediates auxin and cytokinin signals in the control of cell proliferation. Curr. Biol. **13:** 837-842.

Simmonds, D.H. (1994). Mechanism of induction of microspore embryogenesis in Brassica napus: significance of the preprophase band of microtubules in the first sporophytic division. In Biomechanics of Active Movement and Division of Cells (Springer), pp. 569-574.

Simmonds, D.H., and Keller, W.A. (1999). Significance of preprophase bands of microtubules in the induction of microspore embryogenesis of *Brassica napus*. Planta **208:** 383-391.

Soriano, M., Li, H., and Boutilier, K. (2013). Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reprod. **26:** 181-196.

Stasolla, C., Belmonte, M.F., Tahir, M., Elhiti, M., Khamiss, K., Joosen, R., Maliepaard, C., Sharpe, A., Gjetvaj, B., and Boutilier, K. (2008). Buthionine sulfoximine (BSO)-mediated improvement in cultured embryo quality in vitro entails changes in ascorbate metabolism, meristem development and embryo maturation. Planta **228:** 255-272.

Stone, S.L., Braybrook, S.A., Paula, S.L., Kwong, L.W., Meuser, J., Pelletier, J., Hsieh, T.-F., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2008). *Arabidopsis* LEAFY COTYLEDON2 induces maturation traits and auxin activity: implications for somatic embryogenesis. Proc. Natl. Acad. Sci. USA **105:** 3151-3156.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature **403:** 41-45.

Su, Y.H., Zhao, X.Y., Liu, Y.B., Zhang, C.L., O' Neill, S.D., and Zhang, X.S. (2009). Auxin-induced *WUS* expression is essential for embryonic stem cell renewal during somatic embryogenesis in Arabidopsis. Plant J. **59:** 448-460.

Suhasini, K., Sagare, A.P., Sainkar, S.R., and Krishnamurthy, K.V. (1997). Comparative study of the development of zygotic and somatic embryos of chickpea (*Cicer arietinum* L.). Plant Sci. **128:** 207-216.

Supena, E.D.J., Winarto, B., Riksen, T., Dubas, E., Van Lammeren, A., Offringa, R., Boutilier, K., and Custers, J. (2008). Regeneration of zygotic-like microspore-derived embryos suggests an important role for the suspensor in early embryo patterning. J. Exp. Bot. **59:** 803-814.

Tan, X., Calderon-Villalobos, L.I.A., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M., and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446:** 640-645.

Tanaka, M., Kikuchi, A., and Kamada, H. (2008). The Arabidopsis histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. Plant Physiol. **146:** 149-161.

Tang, X., Hou, A., Babu, M., Nguyen, V., Hurtado, L., Lu, Q., Reyes, J.C., Wang, A., Keller, W.A., and Harada, J.J. (2008). The Arabidopsis BRAHMA chromatin-remodeling ATPase is involved in repression of seed maturation genes in leaves. Plant Physiol. **147:** 1143-1157.

Taylor, D.C., Weber, N., Underhill, E.W., Pomeroy, M.K., Keller, W.A., Scowcroft, W.R., Wilen, R.W., Moloney, M.M., and Holbrook, L.A. (1990). Storage-protein regulation and lipid accumulation in microspore embryos of *Brassica napus* L. Planta **181:** 18-26.

Teale, W.D., Paponov, I.A., and Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. Nat. Rev. Mol. Cell Biol. **7:** 847-859.

Telmer, C.A., Simmonds, D.H., and Newcomb, W. (1992). Determination of developmental stage to obtain high-frequencies of embryogenic microspores in *Brassica napus*. Physiol. Plant. **84:** 417-424.

Testillano, P., Georgiev, S., Mogensen, H., Coronado, M., Dumas, C., Risueno, M., and Matthys-Rochon, E. (2004). Spontaneous chromosome doubling results from nuclear fusion during in vitro maize induced microspore embryogenesis. Chromosoma **112:** 342-349.

Tian, L., and Chen, Z.J. (2001). Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. Proc. Natl. Acad. Sci. USA **98:** 200-205.

Tian, L., Fong, M.P., Wang, J.J., Wei, N.E., Jiang, H., Doerge, R., and Chen, Z.J. (2005). Reversible histone acetylation and deacetylation mediate genome-wide, promoter-dependent and locus-specific changes in gene expression during plant development. Genetics **169:** 337-345.

Tiwari, S.B., Wang, X.-J., Hagen, G., and Guilfoyle, T.J. (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. Plant Cell **13:** 2809-2822.

Touraev, A., Vicente, O., and Heberle-Bors, E. (1997). Initiation of microspore embryogenesis by stress. Trends Plant Sci. **2:** 297-302.

Touraev, A., Pfosser, M., and Heberle-Bors, E. (2001). The microspore: a haploid multipurpose cell. Adv. Bot. Res. **35:** 53-109.

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

Touraev, A., Indrianto, A., Wratschko, I., Vicente, O., and HeberleBors, E. (1996b). Efficient microspore embryogenesis in wheat (*Triticum aestivum L*) induced by starvation at high temperature. Sex. Plant Reprod. **9:** 209-215.

Treml, B.S., Winderl, S., Radykewicz, R., Herz, M., Schweizer, G., Hutzler, P., Glawischnig, E., and Ruiz, R.A.T. (2005). The gene *ENHANCER OF PINOID* controls cotyledon development in the *Arabidopsis* embryo. Development **132:** 4063-4074.

Turck, F., Roudier, F., Farrona, S., Martin-Magniette, M.-L., Guillaume, E., Buisine, N., Gagnot, S., Martienssen, R.A., Coupland, G., and Colot, V. (2007). Arabidopsis TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. PLoS Genet. **3:** e86.

Tykarska, T. (1976). Rape embryogenesis. I. The proembryo development. Acta Soc. Bot. Pol. **45:** 3-16.

Uddenberg, D., Valladares, S., Abrahamsson, M., Sundström, J., Sundås-Larsson, A., and von Arnold, S. (2011). Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor. Planta **234:** 527-539.

Ueda, M., Zhang, Z., and Laux, T. (2011). Transcriptional activation of *Arabidopsis* axis patterning genes *WOX8/9* links zygote polarity to embryo development. Dev. Cell **20:** 264-270.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1997a). ARF1, a transcription factor that binds to auxin response elements. Science **276:** 1865-1868.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1999). Activation and repression of transcription by auxin-response factors. Proc. Natl. Acad. Sci. USA **96:** 5844-5849.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997b). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9:** 1963-1971.

Vernon, D.M., and Meinke, D.W. (1994). Embryogenic transformation of the suspensor in *twin*, a polyembryonic mutant of *Arabidopsis*. Dev. Biol. **165:** 566-573.

Vroemen, C.W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A., and de Vries, S.C. (2003). The *CUP-SHAPED COTYLEDON3* gene is required for boundary and shoot meristem formation in *Arabidopsis*. Plant Cell **15:** 1563-1577.

Wójcikowska, B., Jaskóła, K., Gąsiorek, P., Meus, M., Nowak, K., and Gaj, M.D. (2013). *LEAFY COTYLEDON2* (*LEC2*) promotes embryogenic induction in somatic tissues of Arabidopsis, via *YUCCA*-mediated auxin biosynthesis. Planta **238:** 425-440.

Weijers, D., Schlereth, A., Ehrismann, J.S., Schwank, G., Kientz, M., and Jürgens, G. (2006). Auxin triggers transient local signaling for cell specification in *Arabidopsis* embryogenesis. Dev. Cell **10:** 265-270.

Wendrich, J.R., and Weijers, D. (2013). The Arabidopsis embryo as a miniature morphogenesis model. New Phytol.

Weterings, K., Apuya, N.R., Bi, Y., Fischer, R.L., Harada, J.J., and Goldberg, R.B. (2001). Regional localization of suspensor mRNAs during early embryo development. Plant Cell **13:** 2409-2425.

Whitehouse, I., Flaus, A., Cairns, B.R., White, M.F., Workman, J.L., and Owen-Hughes, T. (1999). Nucleosome mobilization catalysed by the yeast SWI/SNF complex. Nature **400:** 784-787.

Wilen, R.W., Mandel, R.M., Pharis, R.P., Holbrook, L.A., and Moloney, M.M. (1990). Effects of abscisic acid and high osmoticum on storage protein gene expression in microspore embryos of *Brassica napus*. Plant Physiol. **94:** 875-881.

Wiśniewska, J., Xu, J., Seifertová, D., Brewer, P.B., Růžička, K., Blilou, I., Rouquié, D., Benková, E., Scheres, B., and Friml, J. (2006). Polar PIN localization directs auxin flow in plants. Science **312:** 883-883.

Woo, E.J., Marshall, J., Bauly, J., Chen, J.G., Venis, M., Napier, R.M., and Pickersgill, R.W. (2002). Crystal structure of auxin‐binding protein 1 in complex with auxin. EMBO J. **21:** 2877-2885.

Wu, K., Tian, L., Malik, K., Brown, D., and Miki, B. (2000). Functional analysis of HD2 histone deacetylase homologues in *Arabidopsis thaliana*. Plant J. **22:** 19-27.

Xu, C.-R., Liu, C., Wang, Y.-L., Li, L.-C., Chen, W.-Q., Xu, Z.-H., and Bai, S.-N. (2005). Histone acetylation affects expression of cellular patterning genes in the *Arabidopsis* root epidermis. Proc. Natl. Acad. Sci. USA **102:** 14469-14474.

Xu, L., and Shen, W.-H. (2008). Polycomb silencing of *KNOX* genes confines shoot stem cell niches in *Arabidopsis*. Curr. Biol. **18:** 1966-1971.

Yadegari, R., Paiva, G., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, J.L., Fischer, R.L., Harada, J.J., and Goldberg, R.B. (1994). Cell differentiation and morphogenesis are uncoupled in Arabidopsis *raspberry* embryos. Plant Cell **6:** 1713-1729.

Yang, C., Bratzel, F., Hohmann, N., Koch, M., Turck, F., and Calonje, M. (2013). VAL-and AtBMI1-mediated H2Aub initiate the switch from embryonic to postgerminative growth in *Arabidopsis*. Curr. Biol. **23:** 1324-1329.

Yeung, E.C., and Sussex, I.M. (1979). Embryogeny of *Phaseolus coccineus*: The Suspensor and the Growth of the Embryo-Proper *in vitro*. Zeitschrift für Pflanzenphysiologie **91:** 423-433.

Yeung, E.C., and Meinke, D.W. (1993). Embryogenesis in angiosperms: development of the suspensor. Plant Cell **5:** 1371.

Yeung, E.C., Rahman, M.H., and Thorpe, T.A. (1996). Comparative development of zygotic and microspore-derived embryos in *Brassica napus* L. cv Topas. I. Histodifferentiation. Int. J. Plant Sci.**:** 27-39.

Žárský, V., Garrido, D., Říhová, L., Tupý, J., Vicente, O., and Heberle-Bors, E. (1992). Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sex. Plant Reprod. **5:** 189-194.

Zaki, M., and Dickinson, H. (1991). Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Plant Reprod. **4:** 48-55.

Zhang, F.-l., Aoki, S., and Takahata, Y. (2003). RAPD markers linked to microspore embryogenic ability in Brassica crops. Euphytica **131:** 207-213.

Zhang, F., and Takahata, Y. (2001). Inheritance of microspore embryogenic ability in *Brassica* crops. Theor. Appl. Genet. **103:** 254-258.

Zhang, H., Bishop, B., Ringenberg, W., Muir, W.M., and Ogas, J. (2012). The CHD3 remodeler PICKLE associates with genes enriched for trimethylation of histone H3 lysine 27. Plant Physiol. **159:** 418-432.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., and Dean, D.C. (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell **101:** 79-89.

Zhang, J.Z., and Somerville, C.R. (1997). Suspensor-derived polyembryony caused by altered expression of valyl-tRNA synthetase in the *twn2* mutant of *Arabidopsis*. Proceedings of the National Academy of Sciences **94:** 7349-7355.

Zhang, X., Germann, S., Blus, B.J., Khorasanizadeh, S., Gaudin, V., and Jacobsen, S.E. (2007). The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. Nat. Struct. Mol. Biol. **14:** 869-871.

Zhang, Y., Shin, H., Song, J.S., Lei, Y., and Liu, X.S. (2008). Identifying positioned nucleosomes with epigenetic marks in human from ChIP-Seq. BMC Genomics **9:** 537.

Zhang, Y., Wang, A., Liu, Y., Wang, Y., and Feng, H. (2011). Effects of the antiauxin PCIB on microspore embryogenesis and plant regeneration in *Brassica rapa*. Scientia Horticulturae **130:** 32-37.

Zhao, J.-P., Simmonds, D.H., and Newcomb, W. (1996). Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. Planta **198:** 433-439.

Zhao, J., and Simmonds, D.H. (1995). Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. Physiol. Plant. **95:** 304-309.

Zhao, J., Newcomb, W., and Simmonds, D. (2003). Heat-shock proteins 70 kDa and 19 kDa are not required for induction of embryogenesis of *Brassica napus* L. cv. Topas microspores. Plant and cell physiology **44:** 1417-1421.

Zhao, Y. (2010). Auxin biosynthesis and its role in plant development. Annu. Rev. Plant Biol. **61:** 49.

Zhou, W., Hagberg, P., and Tang, G. (2002a). Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspores in spring *Brassica napus*. Euphytica **128:** 27-34.

Zhou, W., Tang, G., and Hagberg, P. (2002b). Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regul. **37:** 185-192.

Zhou, W., Zhu, P., Wang, J., Pascual, G., Ohgi, K.A., Lozach, J., Glass, C.K., and Rosenfeld, M.G. (2008). Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation. Mol. Cell **29:** 69-80.

Zhou, Y., Tan, B., Luo, M., Li, Y., Liu, C., Chen, C., Yu, C.-W., Yang, S., Dong, S., and Ruan, J. (2013). HISTONE DEACETYLASE19 interacts with HSL1 and participates in the repression of seed maturation genes in *Arabidopsis* seedlings. Plant Cell **25:** 134-148.

Chapter 2

Microspore Embryogenesis: Establishment of Embryo Identity and Pattern in Culture

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Abstract

The developmental plasticity of plants is beautifully illustrated by the competence of the immature male gametophyte to change its developmental fate from pollen to embryo development when exposed to stress treatments in culture. This process, referred to as microspore embryogenesis, is widely exploited in plant breeding, but also provides a unique system to understand totipotency and early cell fate decisions. We summarize the major concepts that have arisen from decades of cell and molecular studies on microspore embryogenesis, and put these in the context of recent experiments, as well as literature obtained from the study of pollen and zygotic embryo development.

Introduction

In by far the majority of plants, embryogenesis takes place in the ovule after fusion of the female and male gametes (fertilization), and starts with the formation of the unicellular zygote. The zygote goes through species-specific cell division and histodifferentiation programs to form a morphologically complete embryo, that in its simplest form comprises a shoot and a root meristem, which will produce new plant organs after germination, a hypocotyl (embryonic stem), and one or more cotyledons.

The plant kingdom is characterized by a high level of developmental plasticity, including the ability of plants to form embryos from cells other than the zygote. This phenomenon is referred to as totipotency, and may be expressed as part of the normal development of some plants, as in apomixis (reviewed by Barcaccia and Albertini, 2013), or may be induced in tissue culture. Two major types of *in vitro* totipotency are observed in plants, and are distinguished by the origin of the explant. Somatic embryogenesis is induced from vegetative tissues, and generates plants of the same ploidy and genetic composition as the donor plant (Zimmerman, 1993; Gaj, 2001; 2004; George et al., 2008). Another form of totipotency is gametophytic embryogenesis, in which either male or female gametes or their associated accessory cells are induced to form embryos (Reynolds, 1997; Bohanec, 2009; Seguí-Simarro, 2010). These cells are derived post-meiotically, therefore the embryos that are produced in culture represent the haploid segregant progeny of the parent plant. In general, haploid embryo induction from the developing male gametophyte is more commonly applied and studied than from the female gametophyte. This is in part due to the large number of male gametophytes contained in a single anther compared to the single female gametophyte per

ovule, and in part due to the ease with which anthers and pure populations of developing male gametophytes can be isolated. In this review, we focus on haploid embryogenesis from the immature male gametophyte as one form of plant totipotency. Many different terms have been used to describe this form of gametophytic embryogenesis, including androgenesis, microspore embryogenesis and pollen embryogenesis. Here we use the more commonly used term 'microspore embryogenesis' to refer to the *in vitro* culture of the immature male gametophyte, regardless of the developmental stage of the cells that form embryos.

The haploid embryos produced through microspore embryogenesis can be germinated and grown into mature plants, but these plants are sterile due to their inability to produce gametes with a balanced chromosome number after meiosis. Chromosome doubling, which occurs either spontaneously in culture or after the application of chromosome doubling agents such as colchicine, restores the ploidy level and fertility of the derived plant (reviewed by Castillo et al., 2009).

Chromosome doubling of haploid embryos produces a plant that is homozygous at each locus in a single generation. These so-called doubled-haploid (DH) plants have been extensively exploited in plant breeding programs to increase the speed and efficiency with which homozygous lines can be obtained (reviewed in Germanà, 2006; Forster et al., 2007). DH technology is traditionally used to genetically fix parental lines for F1 hybrid production, for rapid introgression of new traits through backcross conversion and to develop immortalized molecular mapping populations. DH technology is also being used to fix traits obtained through transformation and mutagenesis, to simplify genome sequencing by eliminating heterozygosity, and for reverse breeding (Dirks et al., 2009; Ferrie and Möllers, 2011).

The utilization of microspore embryogenesis as a biotechnology tool has been extended to a relatively diverse range of plants (Maluszynski et al., 2003; Ferrie and Caswell, 2011). The ability to form haploid embryos is highly species and genotype dependent, therefore protocols need to be developed or fine-tuned on a case-by-case basis. The decisive tissue culture parameter required to induce embryogenic growth is the application of a stress treatment, usually temperature, nutrient or osmotic stress, either alone or in combination (reviewed by Shariatpanahi et al., 2006a; Islam and Tuteja, 2012). Although DH production is widely exploited, there are often one or more bottlenecks that need to be overcome before

an efficient system can be established for a specific crop or genotype. The major bottlenecks in DH production are the lack or low efficiency of haploid embryo induction and the poor conversion of embryos to seedlings (Germanà, 2006), and in cereals, the high frequency of albino plants (reviewed by Kumari et al., 2009; Torp and Andersen, 2009). Even though the use of microspore embryogenesis has been extended to many plant families (Ferrie et al., 2011; Seguí-Simarro et al., 2011; Ferrie, 2013) there are still species of agronomic (tomato, cotton) or scientific relevance (arabidopsis) that remain recalcitrant to this process.

The regenerative competence of plant cells is widely exploited at a practical level, but a deeper mechanistic understanding of the molecular basis for plant totipotency is lacking. Many studies have focused on understanding the cellular and molecular basis of microspore embryogenesis, however the mechanism underlying this cell fate change is still largely unknown. Historically, two dicot plants (*Brassica napus* and tobacco) and two monocot plants (barley and wheat) have served as models for these studies. This review will focus on the recent advances that have been made in understanding the developmental and molecular changes that take place during microspore embryogenesis in these model systems, and will use the knowledge gained from studies on other stages of plant development as a framework to better understand this process. First, we will address the commonly reported cellular changes associated with the establishment of embryo cell fate and evaluate their validity across species and culture conditions. Next, we will discuss how haploid embryos histodifferentiate; specifically what is known about the establishment of polarity, with emphasis on the importance of exine rupture as a positional clue, and the processes that influence meristem maintenance during culture. Finally, the studies on the molecular changes during microspore embryo induction will be put in context of male gametophytic development. Overall, the current perspective on microspore embryo initiation presents a landscape in which several routes can lead to the same final destination. This intrinsic variability needs to be taken into consideration when trying to understand the basis of this developmental switch.

Embryo fate determination *in vitro*

The male gametophyte or pollen grain is a two to three-celled structure. Male gametophyte development is initiated after meiotic division of the pollen mother cell. The four products of meiosis, the microspores, each undergo two mitotic divisions to form the

mature trinucleate male gametophyte. The first mitotic division is pollen mitosis I (PMI), where the unicellular microspore (Fig. 1a) divides asymmetrically to form a large vegetative cell and a small generative cell (Fig. 1b). The vegetative cell arrests in the G1 phase (Bino et al., 1990), while the generative cell divides at pollen mitosis II (PMII) to produce a pollen grain with two sperm cells and a vegetative cell (Borg et al., 2009). Depending on the species PMII can take place inside of the anther, or during pollen germination (Reynolds, 1997).

The pollen grain is a terminally differentiated structure, but can be induced to continue dividing and form haploid embryos in culture. Sunderland and Evans (1980) and Raghavan (Raghavan, 1986) identified five major pathways that are considered to support embryo development. Multinucleate structures can be generated by division of the uninucleate microspore (B pathway), or in young pollen grains by division of the vegetative cell and/or generative cell (A and E pathways). In some plants nuclear fusion between the vegetative cell and the generative cell prior to division (C pathway), as well as the initial formation of a syncytium (D pathway) has also been described. Sporophytic structures formed by division of the microspore are commonly found in *B. napus* (Zaki and Dickinson, 1991), wheat (Indrianto et al., 2001), barley (Pulido et al., 2005) and tobacco (Sunderland and Wicks, 1971). Sporophytic development through division of the vegetative cell accompanied by generative cell degeneration is also common (Sunderland and Wicks, 1971; Sunderland, 1974; Fan et al., 1988; Reynolds, 1993). Reports of multicellular structures comprised of only generative-like cells are scarce, although multinucleate structures comprised of both generative-like and vegetative-like nuclei can be observed in various species including *B.napus* (Fan et al., 1988), soybean (Kaltchuk-Santos et al., 1997), wheat (Szakács and Barnabás, 1988; Reynolds, 1993), barley (González and Jouve, 2005) and pepper (González-Melendi et al., 1996; Kim et al., 2004). These different pathways often coexist in the same cultures at varying frequencies depending on the species, the stage of male gametophyte development and the stress treatment (Custers et al., 1994; Kasha et al., 2001). It is not clear whether all of these pathways lead to the formation of viable embryos. For example in wheat it was suggested that symmetric divisions (equally sized nuclei) of the immature gametophyte (Fig. 1e) would preferentially lead to embryo formation while sporophytic structures containing both generative and vegetative like-nuclei would preferentially form callus (Szakács and Barnabás, 1988). However, strong evidence to support this conclusion in this and other species is

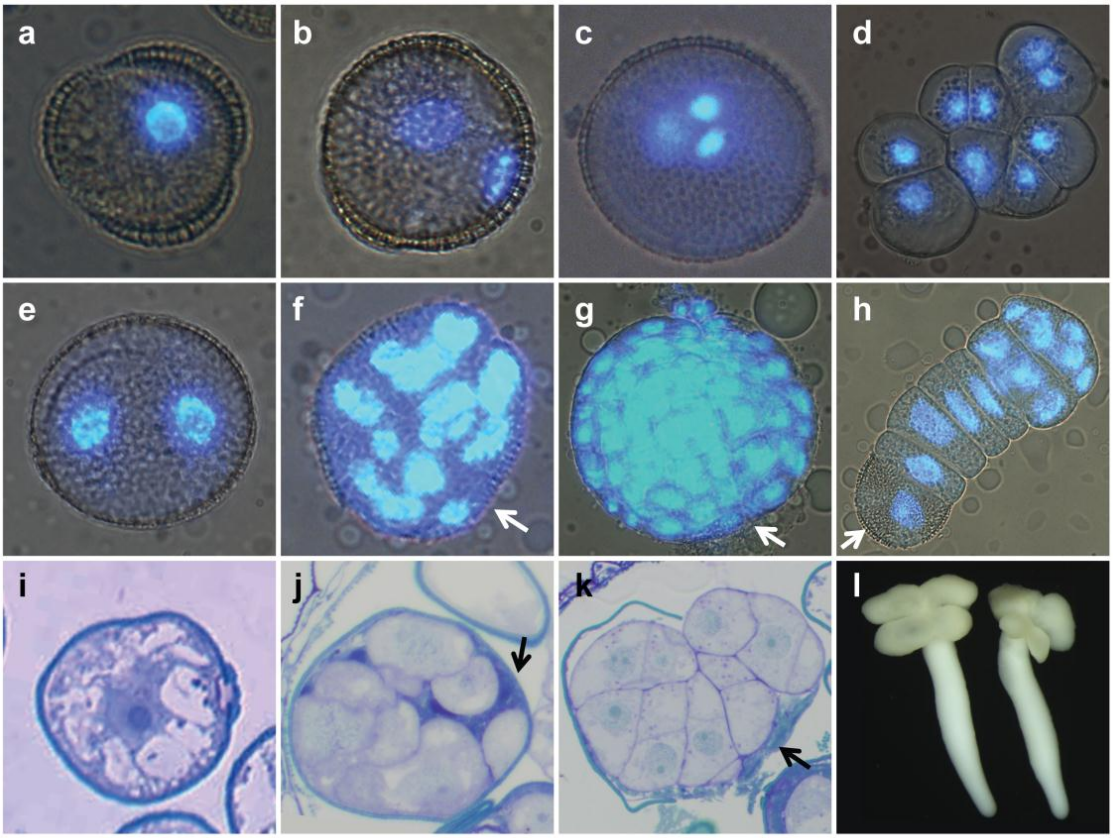

Fig. 1. Developmental pathways observed in *B. napus* and *Triticum aestivum* microspore culture.

(a-c) Male gametophyte development in *B. napus*. (a) Microspore; (b) binucleate pollen with a large vegetative nucleus (vg) and a smaller generative nucleus (g); and (c) trinucleate pollen with a vegetative nucleus and two smaller sperm nuclei (s).

(d-l) Sporophytic structures in *B. napus* (d-h, and l) and wheat (*T. aestivum*) (i-k). (d) Callus-like structure; (e) symmetrically divided microspore with two equally-sized nuclei; (f) multinucleate structure lacking clear organization that is still enclosed within the exine; (g) globular-stage embryo with a well-defined protoderm; (h) suspensor-bearing embryo; (i) star-like morphology after stress treatment; (j) multicellular structure with two distinct domains; (k) multicellular structure breaking out of the exine and (l) microspore-derived embryo at the cotyledon stage. The nuclei in a-h are stained with the nuclear dye 4', 6-diamidino-2-phenylindole (DAPI). White arrows indicate the localization of the exine remnants. Black arrows indicate the small generative-like domain in wheat.

lacking, and the contribution of the different division pathways to the formation of embryos or other types of developmental is not known.

In most species, the stages of pollen that is most responsive for embryo induction are just before or just after PMI, although the exact window of competence is species and even genotype specific (Raghavan, 1986; Bhowmik et al., 2011). After PMII the pollen grain enters a highly specialized transcriptional program that is different from that of both the

microspore/binucleate pollen grain and other sporophytic tissues (see below) (Honys and Twell 2003). These differentiated pollen grains undergo less cell death in culture, most likely because they are more stress resistant than microspores (Thakur et al., 2010), but at the same time they are more resistant to embryogenic induction. A compromise between a low degree of differentiation and stress resistance might be necessary to induce embryogenesis. Alternatively, the competence for embryo induction around PMI could be explained by the ability of the microspore or immature pollen grain cell to divide; microspores near PMI can proceed with division under stress, while younger and older stages cannot, respectively, enter or re-enter the division phase (Reichheld et al., 1999; Giménez-Abián et al., 2004). It is interesting to note that culture conditions also affect the optimal stage that is responsive to induction. For example, anther culture has recurrently been shown to require earlier stages of microspore development than isolated microspore culture (Duijs et al., 1992; Hoekstra et al., 1992). Anther tissues could provide a better environment in which microspores at early stages can develop, by providing nutrients and protection against stress. The anther wall has been proposed to isolate the microspores from the culture medium and delay the timing of induction, necessitating the use of earlier stages as starting material (Hoekstra et al., 1992; Salas et al., 2012). Lastly, microspore isolation represents an added physical stress compared to anther culture (Shariatpanahi et al., 2006b) and therefore might be more effective for late stages of pollen development that require a more intense stress treatment (Binarova et al., 1997).

Microspore embryos are formed in most species by a series of randomly oriented divisions within the surrounding exine wall. The exact point of commitment to embryo development remains unclear, therefore the initial stages are often referred to as sporophytic growth (Fig. 1e), while multicellular, compact structures enclosed in the exine are referred to as both sporophytic structures or embryos (Fig. 1f). Upon rupture of the surrounding exine, a globular embryo is released that comprises a multicellular cluster of cells, with no evident organization and little similarity to its zygotic counterpart, with the exception of a well-defined protoderm (Fig. 1g). The formation of the protoderm is considered a marker for embryo formation (Telmer et al., 1995), and at this point compact structures with a protoderm are normally referred to as embryos, embryoids or embryo-like structures (ELS). Eventually these structures develop into histodifferentiated embryos that contain all the tissues and organs found in zygotic embryos produced *in planta* (Yeung et al.,

1996; Ilić-Grubor et al., 1998). Most embryos are globular in shape without clear apical-basal poles and lack a suspensor structure, or have a rudimentary suspensor formed by few cells. In *B. napus* it is possible to obtain microspore embryos that show a similar, highly organized pattern of cell division as zygotic embryos. In this pathway, a suspensor-like filament is formed by repeated transversal divisions of the microspore, followed by the formation of the embryo proper at the distal end of the suspensor (Fig. 1h). The production of this type of embryo has been optimized in *B. napus* (Joosen et al., 2007; Supena et al., 2008; Prem et al., 2012).

Not all the cultured microspores undergo sporophytic development, and of the microspores that initially switch to sporophytic growth, only a small percentage is able to form embryos. For example, in the model *B. napus* line Topas DH4079, around 40% of the initial population divides sporophytically, while the remaining 60% has a gametophytic identity. The final embryo yield is much lower than the initial 40% sporophytically-divided structures (usually around 5 to 10%). The majority of sporophytic structures stop growing after a few divisions and die, or form callus-like structures that also eventually die (Fan et al., 1988; Telmer et al., 1995; Fig. 1d). In cereals, a high percentage of the microspores divide sporophytically, but form callus rather than embryos (Olsen, 1987; Fadel and Wenzel, 1990; Castillo et al., 2000; Massonneau et al., 2005).

Changes in cellular organization

The main problem associated with defining cellular and morphological traits related to microspore embryogenesis is the heterogeneity of responses observed in culture. As mentioned above, after the stress treatment used to induce embryogenesis, many microspores arrest, divide sporophytically or continue gametophytic development. The microspores that divide sporophytically have different fates; some stop development after a few divisions, some form callus-like structures, and only a small percentage form embryos. Classical cell biology studies have helped to define some of the cellular characteristics of embryogenic cells, although a direct link between cellular changes and cell fate is difficult to establish as these studies are invariably performed on fixed material (Zaki and Dickinson, 1991; Simmonds and Keller, 1999). A few studies have followed the development of microspore cultures using time-lapse imaging and have provided a clearer, although often contradictory picture of the traits that characterize embryogenic microspores and the early

events during embryo induction, as described below (Indrianto et al., 2001; Maraschin et al., 2005b; Daghma et al., 2012; Tang et al., 2013).

The microspores of most species are competent to form an embryo around PMI. At this stage microspores are vacuolated and have a peripherally-located nucleus. It has been proposed that one of the first effects of stress treatments on cultured microspores is the rearrangement of the cytoskeleton, with the displacement of the nucleus to the centre of the cell and the formation of a preprophase band of microtubules (which is absent during normal pollen development) that marks the plane of division (Telmer et al., 1993; Simmonds and Keller, 1999). The application of chemical agents, such as colchicine, cytochalasin D or n-butanol has shown that the rearrangement of the microtubule and actin networks plays a major role in cell fate decisions, since disruption of these networks enhances or is sufficient to trigger embryo formation in the absence of a stress treatment (Zaki and Dickinson, 1991; Szakács and Barnabás, 1995; Zhao et al., 1996; Gervais et al., 2000; Soriano et al., 2008). These cytoskeletal rearrangements drive the displacement of the nucleus to the centre of the cell, resulting in a star-like morphology in which the central nucleus is surrounded by cytoplasmic strands radiating away from the nucleus (Gervais et al., 2000). This star-like morphology has been described in several model systems, and is considered the first sign of embryogenic induction (reviewed by Maraschin et al., 2005a). Live cell imaging of immobilized microspores in wheat and barley showed that a star-like morphology is associated with cell division (Indrianto et al., 2001; Maraschin et al., 2005b), but is not always a reliable marker of embryogenesis, since it can also be observed in cultured microspores that do not form embryos (Maraschin et al., 2005b; Daghma et al., 2012; Żur et al., 2013). Maraschin et al. (2005b) related embryo formation with a subpopulation of microspores in which a star-like morphology appeared later than the majority of the microspores in culture, while Daghma et al. (2012) showed that the star-like morphology can be followed by PMI and starch grain filling, which are both characteristics of pollen development.

Another cellular marker that is often associated with embryo induction is an initial symmetric division of the microspore (Fig. 1e) or the vegetative nucleus of the binucleate pollen grain. The occurrence of this type of division has been reported in a wide range of monocots and dicot species including *B. napus* (Zaki and Dickinson, 1990; Telmer et al., 1993), tobacco (Sunderland and Wicks, 1971), wheat (Indrianto et al., 2001) and barley

(Pulido et al., 2005), and has been correlated with the positive effect of some inducing treatments on embryogenesis, including the application of antimicrotubule agents or heat stress (Zaki and Dickinson, 1991; Szakács and Barnabás, 1995). An initial symmetric division is a recurrent observation in embryogenic microspore cultures, unfortunately, there is no reliable data that correlates the occurrence of a symmetric division with the embryonic potential or embryo development, especially in cereal species (Barnabás et al., 1999; González and Jouve, 2005). Recently, time-lapse imaging studies in *B. napus* showed that both symmetric and asymmetric divisions can support embryo growth, indicating that cell fate and division symmetry are not tightly coupled (Tang et al., 2013). In agreement with this, pollen that undergoes a symmetric division shows defects in the specification of the generative cell, but not a change in pollen cell fate *per se* (Tanaka and Ito, 1981; Eady et al., 1995; Touraev et al., 1995; Twell et al., 1998).

Recently it was shown that embryogenic structures in *B. napus* undergo autophagy and cytoplasmic remodeling (Corral-Martínez and Seguí-Simarro, 2012). This massive excretion of cell material in embryogenic microspores in *B. napus*, together with the specific up-regulation of the 26S proteasome system found in barley embryogenic microspores (Maraschin et al., 2006), highlights the importance of the remodeling of cellular content as an essential first step toward elimination of gametophytic organization and progression to a new cell fate.

In general, the classical markers associated with embryogenic microspores, such as a star-like morphology or an initial symmetric division cannot be considered reliable enough for early identification of the microspores that will form embryos. Moreover, the use of these morphological markers in low responding genotypes is challenging because it requires the initial screening of an enormous amount of cells (Daghma et al., 2012). Other morphological differences that have been correlated with embryogenic growth, including a thin inner layer of the pollen wall (intine) and lack of amyloplasts are difficult to confirm using light microscopy and time-lapse imaging (Zaki and Dickinson, 1991; Telmer et al., 1995; Maraschin et al., 2005b). The combination of cell tracking with the use of vital stains to visualize cell viability, nuclear morphology or other cellular processes would be a valuable tool to identify early events of embryo induction. Likewise, the information generated on the molecular changes that take place in various microspore culture systems can be used as a starting point to generate reporter lines in which fluorescent reporter proteins can be tracked in real time.

Developmental fates

In *B. napus*, haploid embryo formation is characterized by repeated randomly-oriented divisions inside the exine. The multicellular cluster that develops continues dividing until the pollen wall stretches and breaks, releasing a globular structure (Fig. 1f, g). In addition to these randomly divided embryo clusters with no distinct apical and basal domains, the appearance of embryos with clear apical-basal polarity, in the form of an apical embryo proper and a distal suspensor-like structure, was occasionally reported (Hause et al., 1994; Ilić-Grubor et al., 1998; Yeung, 2002). These suspensor-like structures comprise clusters of larger cells, short rudimentary filaments, or uniseriate filaments attached to the root pole of the embryo. Recently a microspore culture system was developed in *B. napus* cv. Topas DH4079 in which a high frequency of embryos bearing a suspensor structure could be obtained (Joosen et al., 2007; Supena et al., 2008). This system uses a milder and shorter stress treatment and produces a higher frequency of embryos with long uniseriate suspensor, as in zygotic embryos of *B. napus*. These embryos are initiated by multiple transverse divisions that protrude out of the exine through an aperture or furrow, and that continue dividing outside of the exine wall to form a file of cells. The distal cell divides longitudinally and produces the embryo proper (Fig. 1h).

The formation of a suspensor is important in the development of zygotic embryos to position the embryo inside of the seed, transport nutrients from the endosperm, and provide hormones to support embryo growth (Yeung and Meinke, 1993). Moreover, it was shown that early patterning in microspore-derived embryos that contain a suspensor is more similar to that of zygotic embryos, pointing to a novel function of the suspensor in supporting early cellular patterning in the embryo proper. The occurrence of suspensor-bearing embryos has also been reported in microspore embryos of wheat (Rybczynski et al., 1991), but in monocots the morphology of the suspensor in zygotic embryos is generally more unorganized than in arabidopsis and *B. napus* (Bommert and Werr, 2001; Guillon et al., 2012), which could make it difficult to identify them *in vitro*.

The occurrence of callus-like growth often takes place side-by-side with embryo formation (Fan et al., 1988; Telmer et al., 1995; Custers et al., 1999; Massonneau et al., 2005). In tobacco, multicellular structures were described that emerge prematurely from the exine and stop growing or develop into callus (Sunderland and Wicks, 1971). At least two types of disorganized sporophytic structures have been described in *B. napus.* One type of

disorganized structure has a high lipid and starch content and a thick intine, and stops dividing inside of the exine or just after it protrudes from the exine. The other type is comprised of loosely connected masses of large, multinucleate cells that eventually stop dividing (Fan et al., 1988; Telmer et al., 1995). In maize and barley some microspores divide to produce embryogenic calli with varying degrees of regenerability (Stirn et al., 1995; Massonneau et al., 2005). The cellular fate of these callus-like structures, whether they are initially embryogenic, gametophytic or have mixed identity is not known. In general, it remains unclear whether callus and other cell types observed in microspore culture are formed because the initial divisions lose their embryogenic capacity, as in eggplant (Corral-Martínez and Seguí-Simarro, 2012), or if these types of divisions were never embryogenic.

The two distinct forms of sporophytic development corresponding to embryo and callus formation can be differentiated in tobacco and *B. napus* microspore culture using a *35SCaMV::GUS* reporter (Custers et al., 1999). The *35S* promoter is expressed during the vegetative phase of development, but it is not active during male gametophyte development or during early embryo growth before the heart stage. Therefore the expression of this reporter provides a means to differentiate sporophytic microspore divisions that are not committed to the embryogenic pathway. Accordingly, GUS activity driven by the *35S* promoter marked callus structures that did not develop into embryos in a low responding cultivar of *B. napus*, while it was absent in embryogenic structures. In tobacco, *35SCaMV::GUS* reporter marked an early stage of sporophytic development prior to embryo development. This suggests that the establishment of embryogenesis could take place by different developmental pathways, with a more direct switch in *B. napus* and an intermediate callus stage in tobacco.

Polarity establishment and histodifferentiation

Embryogenic microspores show variability in their ability to undergo further growth and differentiation. The development of high quality, histodifferentiated embryos with functional meristems is of major importance for the regeneration of DH plantlets, and can be a limiting step in embryo production in some species and genotypes. The most important steps in embryo formation are 1) the establishment of apical-basal polarity, 2) the acquisition of radial polarity and formation of three main tissue layers (epidermis, cortex and endodermis) by periclinal divisions, and 3) the transition to bilateral growth (with one plane

of bilateral symmetry in monocots and two in dicots), characterized by outgrowth of the cotyledons (dicots) or scutelum (monocots) and the establishment of the shoot apical meristem (Bommert and Werr, 2001; Sabelli, 2012).

Cell division and pattern formation during zygotic embryogenesis in plants has been extensively described and studied, particularly in arabidopsis. The organization of the embryo is initially influenced by positional clues that are present prior to fertilization in the female gametophyte. In arabidopsis, the egg cell is already polarized, but briefly loses its polarization upon fertilization (Ueda et al., 2011). Subsequent changes in the organization of the cytoplasm and cell wall after fertilization (Mansfield and Briarty, 1991; Mansfield et al., 1991) give rise to the zygote, which has a vacuolated polar structure (reviewed by Dodeman et al., 1997; Zhang and Laux, 2011). Initially, the zygote elongates and then divides asymmetrically to form a large basal cell that will become the suspensor and the hypophysis, and a smaller apical cell that will form the embryo proper. While cell division and pattern formation in many species is a highly ordered and tightly regulated process, other species undergo less ordered division patterns with more variation in cell division planes, although a suspensor structure is always formed (Maheshwari, 1950). The existence of variable division patterns suggests that cell specification is determined not only by cellular ontogeny but also by cell position, raising the question as to the importance of these controlled divisions for embryo development *per se* (Kaplan and Cooke, 1997).

The importance of the division pattern for zygotic embryo growth is illustrated by the large number of arabidopsis mutants that show altered cell division during early embryogenesis leading to defects in embryo formation. For example, *knolle* mutants, which lack an epidermal cell layer, cannot grow into a normal embryo and are defective in the establishment of the apical-basal axis (Mayer et al., 1991). Both *fass* and *fackel* mutants are unable to orient their division planes. However, while the *fackel* mutant shows mislocalization of the meristems and is seriously compromised in embryo development (Schrick et al., 2000), in the *fass* mutant, the distinct cell identities are correctly established, although they cannot be identified morphologically. These observations suggest that in some cases, an ordered series of cell division is not required for differentiation (Torres-Ruiz and Jurgens, 1994). In maize, seven out of ten mutants defective in the first asymmetric division of the zygote failed to develop an embryo proper (Sheridan and Clark, 1993). Therefore, even in monocots species, where embryo divisions are not as tightly ordered as in

arabidopsis, early embryo patterning during seed development can be decisive for later embryo development.

The initial morphology of *in vitro* cultured embryos, whether derived from somatic or gametophytic tissue, is generally much less organized than their zygotic counterparts (Yeung et al., 1996; Mordhorst et al., 1997). The initial embryonic divisions of microspore embryos are random and produce a cluster of cells in which different cell types cannot be readily distinguished (Fan et al., 1988; Telmer et al., 1995; Yeung et al., 1996). A suspensor is generally not formed. The development of the globular structure begins to mimic that of zygotic embryos once the embryos break out of the exine, and is marked by the establishment of a protoderm layer (Telmer et al., 1995). In maize, the epidermal marker *LTP2* was specifically expressed in embryo forming structures and not in callus (Massonneau et al., 2005). The formation of a protodermal layer is followed by the enlargement of the apical region, and by a transition stage in which the cotyledons (or the scutellum) start to form (Yeung et al., 1996; Ilić-Grubor et al., 1998; Maraschin et al., 2003). It is not clear how apical-basal polarity is established in microspore embryos, i.e. whether it is established in the microspore, during the first sporophytic divisions inside of the exine, or later in development. In somatic embryos, the surrounding tissues (when present) can provide positional clues, but polarity can also be established in the absence of such tissue. Also, gradients of exogenously applied plant hormones can be established and direct embryo growth and division (Friml et al., 2003). Microspore embryos can develop in the absence of external hormones and sporophytic tissues. The question then arises as to how these unorganized structures form a complete embryo in the absence of an initial formative division and without a supporting suspensor or external positional clues.

Pre-existing polarity cues

In contrast to zygotic embryos, the first embryogenic division in microspore culture is often symmetric (Simmonds and Keller, 1999; Zhang and Laux, 2011). It was proposed by Hause et al. (1993) that an initial asymmetric cell division was not required in microspore embryogenesis because of the high degree of polarization that is already present in the microspore. In cereals, microspores are polarized due to the presence of a single round aperture in the pollen wall. In agreement with this observation, in cereals, early embryogenic multicellular structures contained within the exine are often characterized by

two heterogeneous cell domains; a smaller domain comprised of small, dense cells, and a larger domain comprised of larger cells (Bonet and Olmedilla, 2000; Magnard et al., 2000; Testillano et al., 2002; Maraschin et al., 2005c; Dubas et al., 2010). In maize, the large domain shows similarity to endosperm, including a coenocytic organization with incomplete cell walls, synchronous cell division, vacuolated cytoplasm and starch granules (Testillano et al., 2002). Endosperm-specific gene expression was detected in these structures, but was not restricted to the endosperm-like domains (Massonneau et al., 2005). The microspores of dicots like *B. napus* also show polarized development, with a central vacuole and the nucleus localized to the periphery. However, unlike cereals, embryogenic structures in *B. napus* are usually uniform clusters of cells in which no distinct domains can be distinguished (Fan et al., 1988; Joosen et al., 2007).

The formation of suspensors in *B. napus* could arise due to pre-existing polarity factors in the microspore that remain after exposure to a mild stress (Supena et al., 2008). In microspores subjected to a longer and stronger heat stress, polarity clues from the microspore would be erased and result in symmetric division of the microspore and the formation of randomly divided structures. It was also suggested by Straatman et al. (2001) that suspensor- like structures result from aberrant growth induced by the early rupture of the microspore exine wall. Interestingly, recent work by Tang et al. (2013) suggests that the partial breakage of the exine increases the formation of suspensor-bearing embryos. Therefore it would be reasonable to think that polarity clues derived from specific characteristics of the microspores (i.e. cell wall properties, remnants of cellular organization), and/or by the early rupture of the pollen wall, could trigger the formation of polarized suspensor structures.

Exine rupture

Exine rupture is an important step in microspore embryo growth. Most of the sporophytic divisions that fail to form an embryo stop dividing before the exine ruptures (Maraschin et al., 2005c) or when it breaks prematurely (Sunderland and Wicks, 1971; Telmer et al., 1993). Several reports have shown that the site of rupture plays an important role in polarity establishment. Regardless of the species, exine remnants often remain attached to the root pole, suggesting that the apical domain of the embryo coincides with the site of exine rupture (Hause et al., 1993; Ilić-Grubor et al., 1998; Indrianto et al., 2001; Tang et al., 2013).

In *B. napus,* the male gametophyte contains three pollen apertures and two types of exine rupture during microspore culture have been described; type I in which the cells grow and increase volume, protruding out of the apertures and type II in which the structure grows in a uniform way producing the even stretch of the exine (Nitta et al., 1997). It is not known whether preexisting polar growth drives the site of exine rupture or if polarity is established as a consequence of the differential rupture. The first morphological sign of polarity establishment in *B. napus* is the disappearance of starch granules at the site of exine rupture, which will become the future apical pole (Hause et al., 1994). Studies in brown algae (*Fucus*) embryos show that the cell wall provides positional information to establish a polar axis and orient the first cell division plane of the zygote, and that differences in cell wall composition are important for cell fate determination (Belanger and Quatrano, 2000). Localized vesicular secretion is essential for remodeling of the cell wall and for the establishment of polarization in *Fucus*, and has also been shown to be important in vascular plants for polar transport of the morphogen hormone auxin (Belanger and Quatrano, 2000; Geldner et al., 2003). The cell wall changes that characterize the switch to microspore embryogenesis, include the moderate growth of the innermost pecto-cellulosic wall and intine (Telmer et al., 1995; Bonet and Olmedilla, 2000; Schulze and Pauls, 2002; Solís et al., 2008), an increase in pectin esterification (Bárány et al., 2010), and the differential localization of arabinogalactan epitopes (El-Tantawy et al., 2013). These or other changes in the cell wall properties could be important for the ability of induced microspores to develop into embryos and require further study. The role of the cell wall and other structural cell components in the regulation of plant growth is receiving increasing attention, especially in light of their importance as mediators of mechano-stress signaling and the regulation of organ growth (Braybrook and Peaucelle, 2013) and references therein).

Premature exine rupture seems to be detrimental to further embryo growth. However, in *B. napus* suspensor-bearing embryos, the exine ruptures after only a few cell divisions, but the suspensor filament develops and is thought to emerge through one of the pollen apertures. Microspores with a partially broken pollen wall, so-called exine-dehisced microspores (EDM) can be obtained by breaking the exine by physical stress. The EDM elongates and protrudes of out of the ruptured site and often gives rise to the formation of well-developed suspensor embryos (Tang et al., 2013). The orientation of the first division plane in these EDM is predominantly transversal to the axis marked by exine rupture (which

is defined by the remnants of the exine in one extreme of the cell), and therefore it has been proposed that the location of exine rupture determines the division plane via mechanical stress. This work, together with the observations of Hause et al. (1994) show that in *B. napus* the site of exine rupture can direct the polarity axis of the embryo, and points to a role for the pollen wall in microspore embryo organization.

In barley microspores, which have only one aperture, the embryo consistently breaks out of the exine at the side opposite to the aperature. This process has been proposed to be regulated by cell death of the small cell domain, which is localized at the site of rupture (Maraschin et al., 2005c). In barley, the small cell domain has been associated with repeated division of the generative cell and its presence is important to promote exine rupture: homogeneous multicellular structures that lack this domain fail to break the exine and do not develop further (Maraschin et al., 2005c). The question that remains is how this cell death process is regulated, i.e. whether it is the cause or consequence of exine rupture. It would be interesting to determine if PCD is regulated in systems were morphological distinct domains might be absent prior to pollen wall rupture, such as *B. napus*. It is clear that in some cases the establishment of polarity precedes rupture of the microspore wall and determines both the site of the rupture and the orientation of the body axis of the embryo. However there is increasing evidence for the role of the pollen wall in defining the apical-basal axis. The variability that seems to operate in different species should be explored to gain insight into the pathways that lead to plant and embryo polarity and self-organization.

Maintenance of meristem integrity

Once the exine breaks, the main tissue layers of the embryo are formed, which include the protoderm, the procambium and the ground tissue layers that will form, respectively, the epidermis, the vascular tissue and the parenchyma. The apical-basal axis of the embryo is established by the formation of the meristems. Although embryos produced *in vitro* initially develop well-formed meristems, these meristems may degenerate later in culture (Stasolla et al., 2008). Embryos that contain degenerated meristems cannot be converted directly into plants. This degeneration primarily affects the shoot apical meristem (SAM) and is characterized by acquisition of parenchymous features such as the formation of intercellular spaces and vacuolation, as well as loss of meristem identity (Belmonte et al., 2005). (2005; Belmonte et al., 2006) proposed that the degeneration of the meristems

during *in vitro* culture was due to the requirement for a more oxidized environment during late embryo development. In agreement with this hypothesis, abnormalities in SAM organization that are observed in the late phases of microspore embryo development can be rescued by a lower cellular redox state, obtained by chemical inhibition of *de novo* glutathione synthesis (by application of buthionine sulfoximine, BSO), or by treatment with the oxidized form of glutathione (2006; Belmonte et al., 2011). Glutathione and ascorbate are molecules with both oxidized and reduced forms that play a role in the detoxification and scavenging of reactive oxygen species, and in the regulation of the redox cellular state. BSO treatment affects ascorbate metabolism, producing lower ascorbate levels in treated embryos, and activated expression of meristem-specific genes including *ZWILLE*, *SHOOTMERISTEMLESS*, and *ARGONAUTE 1* (Stasolla et al., 2008). The more oxidized environment produced by BSO also reduces the level of ethylene and induces gene expression associated with the embryo maturation phase of zygotic embryo development, including ABA response proteins and late-embryogenic abundant (LEA) proteins. Overall, the change in redox status during embryo development produces a metabolic switch needed for the embryos to reach maturity. This change is proposed to be mediated by an ABA response, since ABA treatment produces similar effects on embryo maturation and conversion frequencies (Belmonte et al., 2006; Ramesar-Fortner and Yeung, 2006).

Enhancement of proper SAM functionality in microspore embryos was also attained by the overexpression of *SHOOT MERISTEMLESS* (*STM*), a Class I knotted-like homeodomain transcription factor that functions in SAM initiation and maintenance (Barton and Poethig, 1993). *STM* overexpression maintains expression of cell cycle machinery genes and characteristics of meristematic cells, while repressing the cell wall modifications typical of cell differentiation (Elhiti et al., 2013). Overexpression of *STM* induced expression of known embryogenesis regulatory genes and also reduced reactive oxygen species (ROS) by the increase in scavenging enzyme activity, and by increased ascorbic acid (Elhiti et al., 2013). Elhiti et al. proposed that STM delays cellular differentiation through a decrease in ROS levels and by reducing cell wall rigidity.

It has been proposed that the maintenance of cellular brassinosteroid levels is required for the formation of functional apical meristems. This view is supported by the increase in the number and quality of microspore-derived embryos upon treatment with externally applied brassinolide, whereas treatment with brassinazole, a brassinosteroid biosynthetic

inhibitor, has the opposite effect (Belmonte et al., 2011). Interestingly, upon brassinazole treatment the ascorbate and glutathione pools in microspore embryos switch toward an oxidized state, supporting a role of brassinosteroids in the regulation of the redox state during embryo development. A role for brassinosteroids in control of the cellular redox state of the SAM during the transition to the maturation phase of development in zygotic embryos has not been described.

Molecular control of haploid embryo induction

The developmental starting point for microspore embryogenesis is the male gametophyte. Therefore, to understand the molecular basis for haploid embryo induction, this change in development must be placed in the context of the normal pathway of pollen development. This comparison is especially important, when one considers that the vast majority of cultured microspores and pollen do not form embryos, but rather continue gametophyte development or arrest and die.

The developmental stage of the immature male gametophyte is a critical factor that influences the embryogenic potential. Transcriptome analyses in arabidopsis (Honys and Twell, 2004) and wheat (Tran et al., 2013) have shown that the transcriptomes of microspores and bicellular pollen are highly similar. Their transcriptomes show little overlap with that of mature pollen, but rather are more similar to those of other sporophytic stages of plant development (Honys and Twell, 2004; Joosen et al., 2007; Whittle et al., 2010; Tran et al., 2013). The microspore transcriptome is characterized by a higher proportion of transcripts encoding structural proteins, as well as proteins involved in translation and metabolism (Whittle et al., 2010). As pollen matures, there is a shift toward expression of fewer, but more highly abundant, pollen-specific transcripts that mainly encode proteins involved in pollen germination and tube growth (Becker et al., 2003; Loraine et al., 2013). The course of male gametophyte development is therefore characterized by a shift toward a higher degree of specialization. The initial similarity between the microspore/bicellular pollen and sporophytic stages of plant development may provide the developmental competence that is needed to switch from gametophytic to sporophytic growth during microspore embryogenesis (Whittle et al., 2010).

Gene expression studies aimed at understanding the molecular basis of microspore embryogenesis have relied on comparison between cultures induced to undergo

embryogenesis and non-induced cultures containing developing pollen. Although these studies have a common goal, it is difficult to develop a common picture of the molecular changes that accompany the switch from pollen development to haploid embryogenesis. Firstly, the available studies are focused on different model species, each induced with one or more treatments, including high temperature stress, nutrient starvation and/or osmotic stress, and each with different starting material e.g. isolated microspores or anthers. Secondly, each of these studies has been performed using different, mainly low-throughput, approaches to identify transcripts of interest, including screening of cDNA libraries (Hosp et al., 2007), sequencing of expressed sequence tags (ESTs, Malik et al., 2007; Tsuwamoto et al., 2007), targeted expression analysis of candidate genes (Sánchez-Díaz et al., 2013), and custom (Maraschin et al., 2006; Joosen et al., 2007) and commercial (Muñoz-Amatriaín et al. 2006) DNA arrays. A third problem is the low embryogenic response of the cultures, although approaches to enrich for embryogenic microspores (Maraschin et al., 2006) or specific sequences (Malik et al., 2007) have been carried out. Given the limitations outlined above, we discuss the major concepts that have emerged from these studies.

Deregulation of pollen development

It is generally assumed that microspore re-programming to embryogenesis is achieved, in part, by repressing gametophytic development. In barley, microspore embryogenesis is induced by exposing cultured anthers to starvation and osmotic stress using mannitol. A highly embryogenic fraction of microspores can be purified by density centrifugation after four days of culture in the anther (Maraschin et al., 2005b). Comparison of the gene expression profiles of this enriched fraction with pollen showed that while pollen development was characterized by the expression of starch biosynthesis genes, the embryogenic microspore fraction showed the opposite trend: a decrease in the expression of starch biosynthesis genes, and an increase in expression of genes involved in sugar and starch hydrolysis (Maraschin et al., 2006). This observation is in agreement with many studies showing that starch accumulates during late pollen development and that its accumulation in microspore culture is detrimental to embryo progression (McCormick, 1993). This data, although limited, also implies that at least some genes involved in pollen development are down-regulated during embryo induction (Maraschin et al., 2006).

In *B. napus*, isolated microspores develop into embryos after exposure to heat stress. The first sporophytic divisions are observed after one to two days of culture, and by five days of culture, globular-shaped multicellular structures are formed that begin to emerge from the surrounding exine. Initially, the vast majority of cells in culture follow the gametophytic pathway, but around five to six days of culture, the pollen grains burst open and die. Microarray analysis has shown that two-day old heat-stressed microspore cultures are highly similar to pollen cultures (Joosen et al., 2007). Only at five days of culture, when the pollen is dead, can genes that are differentially-expressed between pollen and embryogenic microspore cultures be identified by microarray analysis. It was not clear from this analyses whether the gene expression profiles associated with embryogenic microspores at two days of culture were swamped by the highly abundant, stable, late pollen transcripts, or whether both pollen and embryo development coincided in the same cell types. It is possible to identify proteins that are differentially expressed in two day-old induced cultures compared to pollen. Although co-existence of pollen and embryo identities in the same structure cannot be excluded, differential protein expression as early as two days of culture does support the observation that the abundance of late pollen transcripts in the RNA samples is due to the presence of non-translated pollen mRNAs (Mascarenhas, 1990, 1993; Ylstra and McCormick, 1999), and that proteomics may provide a more sensitive approach to identifying totipotency-related pathways. Suspensor-bearing embryos develop much slower than suspensor-less embryos. After eight days of culture, embryos with a long suspensor and a one-to-two celled-embryo proper have developed, while the gametophytic cells are no longer viable. Microarray analysis of these samples show clearly different expression profiles from those of developing pollen, indicating that, at least in this pathway, embryogenic and gametophytic gene expression do not co-exist in the same structures. Malik et al. (2007) showed using hand isolated five-day old embryos that lack suspensors, that both pollen and embryo markers are expressed in the same samples. In support of this, Pulido et al. (2009) have shown that the late pollen gene *PG1* is expressed in few-celled sporophytic structures found in barley cultures, but disappears as sporophytic growth progresses. The question of whether and to what extent active pollen and embryo gene expression occur in parallel in microspore embryos is intriguing, but cannot be answered at this time. Live imaging using rapidly turned-over pollen reporters together with embryo identity reporters will be needed

to determine whether pollen genes are actively expressed in embryogenic microspores or whether these mRNAs are simply remnants of highly stable transcripts.

Establishment of embryo identity

As mentioned above, studies aimed at identifying the early molecular events that accompany haploid embryo induction have been hampered by the presence of highly abundant pollen transcripts. A few studies have identified differentially-expressed sequences using methods to subtract pollen-expressed genes (Joosen et al., 2007; Malik et al., 2007; Tsuwamoto et al., 2007), but these analyses were performed late in the development of the culture, when sporophytic clusters are already present. The study by Maraschin et al. (2006) is to our knowledge the only study that examined gene expression in microspores that were induced to undergo embryogenesis, but had not yet divided. When the expression profile of these cells was compared to microspores and developing pollen, they found evidence for a role for proteolysis, stress response, inhibition of programmed cell death, and signaling pathways in embryo induction, that could be separated from effects of the stress treatment used to induce embryogenesis. Unfortunately, the number of genes examined in this study is small, precluding a more global analysis of these pathways activated during haploid embryo induction.

Two other studies in *B.napus* examined gene expression profiles in embryos at a slightly later stage of development, starting from two to three days of culture, when induced microspores had already gone through a few sporophytic cell divisions (Joosen et al., 2007; Malik et al., 2007). Malik et al. (2007) noted a sharp decrease in the expression of protein synthesis machinery genes at day 3 of microspore culture and associated this drop in expression with a switch to the embryogenic pathway. The observation may be explained by the dominance of late pollen-expressed genes at this stage and the normal decrease in expression of protein machinery genes during late pollen development (Honys and Twell, 2004; Joosen et al., 2007; Whittle et al., 2010). In support of this, expression of protein synthesis machinery genes increased in five and seven-day old cultures (Joosen et al., 2007; Malik et al., 2007), coinciding with the loss of pollen and increase in sporophytic growth. This point highlights the difficulty of analyzing gene expression profiles in highly heterogeneous cultures in which many different developmental pathways co-exist.

While detailed studies on the events prior to embryogenic division are lacking, there is much more known about the expression of early embryo genes in microspore culture, specifically in *B. napus* (Joosen et al., 2007; Malik et al., 2007; Tsuwamoto et al., 2007). Malik et al. (2007) identified a large number *B. napus* ESTs that show strong sequence similarity to known arabidopsis embryo-expressed genes, in particular transcription factor genes. The expression of 24 of these genes was rigorously examined using quantitative RT-PCR in induced and non-induced microspore cultures, during seed development, and during other stages of sporophytic development. Based on these results, they were able to identify a set of genes that are expressed in haploid and zygotic embryo development, but not during pollen development. These genes include *FUSCA3*, *LEAFY COTYLEDON1 (LEC1)*, *LEC2, BABY BOOM (BBM)*, two WUSCHEL- related homeobox (WOX) genes, *WOX2* and *WOX9*, and *ABSCISIC ACID INSENSITIVE3*. Although ESTs for these genes were only detected after seven days of culture their expression could be detected by RT-PCR much earlier, at one to two days of culture, suggesting that embryo cell identity is established as early as the first few sporophytic cell divisions. The utility of a subset of these genes as early markers for embryogenic growth in genotypes differing in their ability to form haploid embryos was also examined. Only the expression of one of the markers, *LEC2*, could distinguish between embryogenic and non-embryogenic cultures at three days, but all of them distinguish the same cultures at seven days. The low correlation with embryo formation is not surprising, as the expression of the marker depends on many factors, including its own expression level, the proportion of embryogenic cells in the culture, and whether the genotype is negatively affected in a pathway in which the marker gene normally functions. Unpublished work from our lab suggests that a LEC1:GFP fusion is specifically expressed in embryos during seed development (Fig. 2a) and marks embryogenic microspores in culture in a poorly-responding genotype as early as three days after the start of culture (Fig. 2b).

The *B. napus* suspensor-embryo system also proved to be a valuable tool to identify early embryo-expressed genes (Joosen et al., 2007). Suspensor-bearing embryos develop more slowly than conventional cultures, so that by the time the embryo proper has reached the two-cell stage the pollen that co-develops in the culture has already died. Comparison of conventional embryos without a suspensor and embryos with a suspensor (few-celled to globular stage embryo proper) identified a set of 43 genes whose expression is significantly upregulated in embryogenic microspore cultures compared to the male gametophyte. The

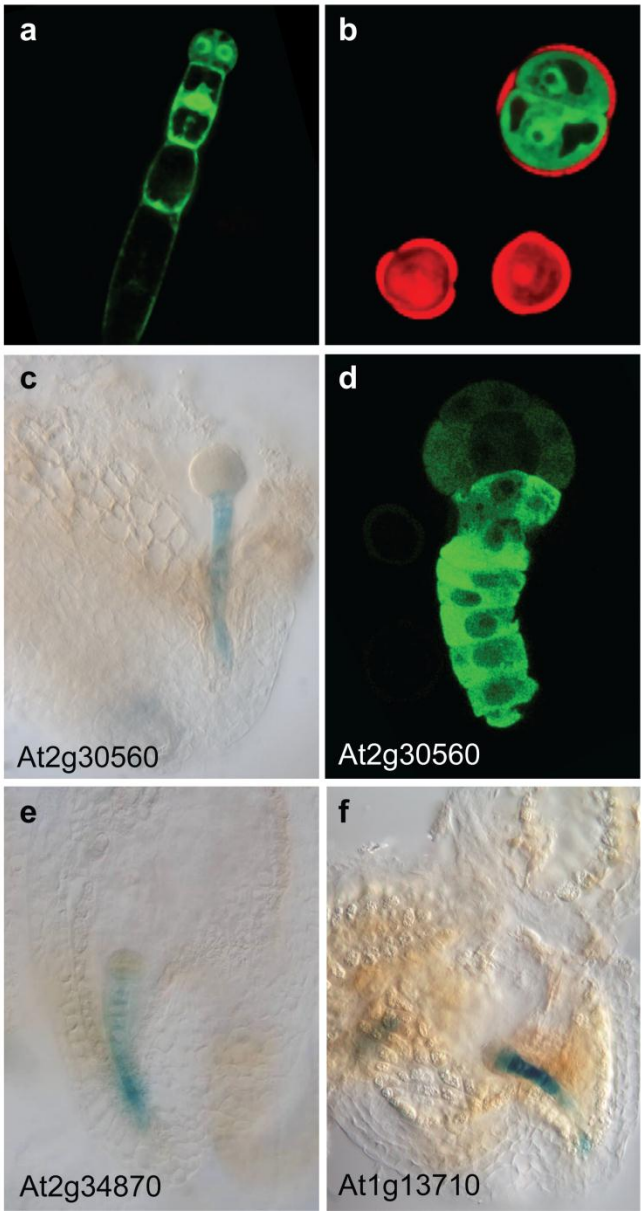

Fig. 2 Expression of suspensor and embryo markers identified in *B. napus* microspore culture

Expression of an arabidopsis *LEC1::LEC1:GFP* reporter in (a) the two-celled embryo proper and suspensor of a *B. napus* zygotic embryo and (b) a sporophytically divided microspore. GFP expression in (a) and (b) is shown in green and autofluorescence in (b) is shown in red. The two smaller microspores in (b) do not show GFP expression. Expression of arabidopsis orthologs of *B. napus* suspensor-expressed genes in arabidopsis zygotic embryos (c, e, f) and a *B. napus* microspore embryo (d). The lines shown in c, e and f are promoter:GUS reporters and the line shown in (e) is a promoter: GFP reporter. The corresponding arabidopsis gene identifier for each reporter is indicated.

suspensor-expression of a number of these genes has been confirmed in arabidopsis and *B. napus* (Fig. 2c-f). This model system for *in vitro* susspensor production offers a novel tool for the isolation and molecular characterization of this poorly accessible tissue.

Based on the above, we can conclude that the molecular activation of the embryo pathway is an early event in haploid embryo induction, at least in *B. napus*. Other studies in barley and tobacco did not specifically describe expression of early embryo-expressed genes in microspore culture (Maraschin et al., 2006; Muñoz-Amatriaín et al., 2006; Hosp et al., 2007). Nonetheless, it is still not clear which gene expression events, if any, precede the activation of embryo gene expression in microspore embryo induction. Ectopic expression of

the *LEC1*, *LEC2* and *BBM* transcription factors in seedlings is sufficient to induce activation of embryo-expression programs, as well as the *de novo* induction of somatic embryo formation (Lotan et al., 1998; Stone et al., 2001; Boutilier et al., 2002). Given the sporophyte-like identity of the microspore and bicellular pollen grain, *de novo* expression of these transcription factors in response to stress could be sufficient to induce a switch to totipotent growth. On the other hand, the expression of embryo markers may simply represent an early, but secondary event that is set in motion by the stress treatment.

Conclusion and perspective

Microspore embryogenesis has been extensively studied, but still the mechanism that drives this process, from the initial embryogenic cell divisions to the formation of histodifferentiated embryos, is not understood. Many of the early cell biological observations on microspore embryo induction are now being revised or even discarded in light of live imaging studies. The picture is even less clear at the molecular level, where different starting materials, type and duration of induction treatment, and gene expression platforms have been used to probe the embryogenic microspore. To proceed further requires a collaborative approach in which live imaging is combined with cell and molecular analyses. The different culture systems need to be stripped down to their simplest elements to facilitate a direct comparison, and high throughput DNA and protein sequencing techniques are needed to identify and compare transcripts in microspores and pollen, as well as in embryogenic- and stressed, non-embryogenic microspores. The identified genes need to be definitively linked to microspore embryogenesis pathway, rather than stress response, using genetic and genomics approaches, such as mutant analysis and time-lapse imaging of candidate and other pathway markers in live cells.

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References

Bárány, I., Fadón, B., Risueño, M.C., and Testillano, P.S. (2010). Cell wall components and pectin esterification levels as markers of proliferation and differentiation events during pollen development and pollen embryogenesis in *Capsicum annuum* L. J. Exp. Bot. **61:** 1159-1175.

Barcaccia, G., and Albertini, E. (2013). Apomixis in plant reproduction: a novel perspective on an old dilemma. Plant reproduction **26:** 159-179.

Barnabás, B., Obert, B., and Kovács, G. (1999). Colchicine, an efficient genome-doubling agent for maize (*Zea mays* L.) microspores cultured in anthero. Plant Cell Rep. **18:** 858-862.

Barton, M.K., and Poethig, R.S. (1993). Formation of the shoot apical meristem in Arabidopsis thaliana: an analysis of development in the wild type and in the shoot meristemless mutant. Development **119:** 823-831.

Becker, J.D., Boavida, L.C., Carneiro, J., Haury, M., and Feijó, J.A. (2003). Transcriptional profiling of Arabidopsis tissues reveals the unique characteristics of the pollen transcriptome. Plant Physiol. **133:** 713-725.

Belanger, K.D., and Quatrano, R.S. (2000). Polarity: the role of localized secretion. Curr. Opin. Plant Biol. **3:** 67-72.

Belmonte, M., Elhiti, M., Ashihara, H., and Stasolla, C. (2011). Brassinolide-improved development of Brassica napus microspore-derived embryos is associated with increased activities of purine and pyrimidine salvage pathways. Planta **233:** 95-107.

Belmonte, M.F., Donald, G., Reid, D.M., Yeung, E.C., and Stasolla, C. (2005). Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). J. Exp. Bot. **56:** 2355-2364.

Belmonte, M.F., Ambrose, S.J., Ross, A.R., Abrams, S.R., and Stasolla, C. (2006). Improved development of microspore-derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism. Physiol. Plant. **127:** 690-700.

Bhowmik, P., Dirpaul, J., Polowick, P., and Ferrie, A.M. (2011). A high throughput *Brassica napus* microspore culture system: influence of percoll gradient separation and bud selection on embryogenesis. Plant Cell Tiss. Org. Cult. **106:** 359-362.

Binarova, P., Hause, G., Cenklová, V., Cordewener, J.H., and Campagne, M.L. (1997). A short severe heat shock is required to induce embryogenesis in late bicellular pollen of Brassica napus L. Sex. Plant Reprod. **10:** 200-208.

Bino, R., Van Tuyl, J., and De Vries, J. (1990). Flow cytometric determination of relative nuclear DNA contents in bicellulate and tricellulate pollen. Ann. Bot. **65:** 3-8.

Bohanec, B. (2009). Doubled haploids via gynogenesis. In Advances in Haploid Production in Higher Plants, A. Touraev, B.P. Forster, and S.M. Jain, eds (Dordrecht: Springer), pp. 35-46.

Bommert, P., and Werr, W. (2001). Gene expression patterns in the maize caryopsis: clues to decisions in embryo and endosperm development. Gene **271:** 131-142.

Bonet, F., and Olmedilla, A. (2000). Structural changes during early embryogenesis in wheat pollen. Protoplasma **211:** 94-102.

Borg, M., Brownfield, L., and Twell, D. (2009). Male gametophyte development: a molecular perspective. J. Exp. Bot. **60:** 1465-1478.

Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A.A., and Miki, B.L. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell **14:** 1737-1749.

Braybrook, S.A., and Peaucelle, A. (2013). Mechano-chemical aspects of organ formation in *Arabidopsis thaliana*: The relationship between auxin and pectin. PloS one **8:** e57813.

Castillo, A., Vallés, M., and Cistué, L. (2000). Comparison of anther and isolated microspore cultures in barley. Effects of culture density and regeneration medium. Euphytica **113:** 1-8.

Castillo, A., Cistué, L., Vallés, M., and Soriano, M. (2009). Chromosome doubling in monocots. In Advances in Haploid Production in Higher Plants, A. Touraev, B.P. Forster, and S.M. Jain, eds (Dordrecht: Springer), pp. 329-338.

Corral-Martínez, P., and Seguí-Simarro, J.M. (2012). Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.). Euphytica **187:** 47-61.

Custers, J.B.M., Cordewener, J.H.G., Nöllen, Y., Dons, H.J.M., and van Lockeren Campagne, M.M. (1994). Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep. **13:** 267-271.

Custers, J.B.M., Snepvangers, S.C.H.J., Jansen, H.J., Zhang, L., and van Lookeren Campagne, M.M. (1999). The 35S-CaMV promoter is silent during early embryogenesis but activated during nonembryogenic sporophytic development in microspore culture. Protoplasma **208:** 257-264.

Daghma, D., Kumlehn, J., Hensel, G., Rutten, T., and Melzer, M. (2012). Time-lapse imaging of the initiation of pollen embryogenesis in barley (*Hordeum vulgare* L.). J. Exp. Bot. **63:** 6017-6021.

Dirks, R., van Dun, K., de Snoo, C.B., van Den Berg, M., Lelivelt, C.L., Voermans, W., Woudenberg, L., de Wit, J.P., Reinink, K., and Schut, J.W. (2009). Reverse breeding: a novel breeding approach based on engineered meiosis. Plant Biotechnol. J. **7:** 837-845.

Dodeman, V.L., Ducreux, G., and Kreis, M. (1997). Zygotic embryogenesis versus somatic embryogenesis. J. Exp. Bot. **48:** 1493-1509.

Dubas, E., Wędzony, M., Petrovska, B., Salaj, J., and Żur, I. (2010). Cell structural reorganization during induction of androgenesis in isolated microspore cultures of Triticale (*xTriticosecale* Wittm.). Acta. Biol. Cracov. Bot. **52:** 73-86.

Duijs, J., Voorrips, R., Visser, D., and Custers, J. (1992). Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica **60:** 45-55.

Eady, C., Lindsey, K., and Twell, D. (1995). The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. Plant Cell **7:** 65-74.

El-Tantawy, A.-A., Solís, M.-T., Da Costa, M.L., Coimbra, S., Risueño, M.-C., and Testillano, P.S. (2013). Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*. Plant reproduction**:** 1-13.

Elhiti, M., Wally, O.S., Belmonte, M.F., Chan, A., Cao, Y., Xiang, D., Datla, R., and Stasolla, C. (2013). Gene expression analysis in microdissected shoot meristems of *Brassica napus* microspore-derived embryos with altered *SHOOTMERISTEMLESS* levels. Planta **237:** 1065-1082.

Fadel, F., and Wenzel, G. (1990). Medium-genotype-interaction on androgenetic haploid production in Wheat. Plant breeding **105:** 278-282.

Fan, Z., Armstrong, K., and Keller, W. (1988). Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma **147:** 191-199.

Ferrie, A., and Caswell, K. (2011). Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tiss. Org. Cult. **104:** 301-309.

Ferrie, A., Bethune, T., and Mykytyshyn, M. (2011). Microspore embryogenesis in Apiaceae. Plant Cell Tiss. Org. Cult. **104:** 399-406.

Ferrie, A.M. (2013). Advances in microspore culture technology: a biotechnological tool for the improvement of medicinal plants. In Biotechnology for Medicinal Plants (Dordrecht: Springer), pp. 191-206.

Ferrie, A.M., and Möllers, C. (2011). Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss. Org. Cult. **104:** 375-386.

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

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical–basal axis of *Arabidopsis*. Nature **426:** 147-153.

Gaj, M.D. (2001). Direct somatic embryogenesis as a rapid and efficient system for in vitro regeneration of *Arabidopsis thaliana*. Plant Cell Tiss. Org. Cult. **64:** 39-46.

Gaj, M.D. (2004). Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul. **43:** 27-47.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003). The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112:** 219-230.

George, E.F., Hall, M.A., and De Klerk, G.-J. (2008). Somatic embryogenesis. In Plant propagation by tissue culture (Dordrecht: Springer), pp. 335-354.

Germanà, M.A. (2006). Doubled haploid production in fruit crops. Plant Cell Tiss. Org. Cult. **86:** 131-146.

Gervais, C., Newcomb, W., and Simmonds, D. (2000). Rearrangement of the actin filament and microtubule cytoskeleton during induction of microspore embryogenesis in *Brassica napus* L. cv. Topas. Protoplasma **213:** 194-202.

Giménez-Abián, M., Rozalén, A., Carballo, J., Botella, L., Pincheira, J., López-Sáez, J., and de la Torre, C. (2004). HSP90 and checkpoint-dependent lengthening of the G2 phase observed in plant cells under hypoxia and cold. Protoplasma **223:** 191-196.

González-Melendi, P., Testillano, P., Ahmadian, P., Fadón, B., and Risueño, M. (1996). New in situ approaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. Eur. J. Cell Biol. **69:** 373-386.

González, J., and Jouve, N. (2005). Microspore development during *in vitro* androgenesis in triticale. Biol. Plant. **49:** 23-28.

Guillon, F., Larré, C., Petipas, F., Berger, A., Moussawi, J., Rogniaux, H., Santoni, A., Saulnier, L., Jamme, F., and Miquel, M. (2012). A comprehensive overview of grain development in *Brachypodium distachyon* variety Bd21. J. Exp. Bot. **63:** 739-755.

Hause, B., Hause, G., Pechan, P., and van Lammeren, A.A.M. (1993). Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol. Int. **17:** 153-168.

Hause, B., van Veenendaal, W.L.H., Hause, G., and van Lammeren, A.A.M. (1994). Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv. Topas. Bot. Acta. **107:** 407-415.

Hoekstra, S., van Zijderveld, M.H., Louwerse, J.D., Heidekamp, F., and van der Mark, F. (1992). Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. **86:** 89-96.

Honys, D., and Twell, D. (2003). Comparative analysis of the Arabidopsis pollen transcriptome. Plant Physiol. **132:** 640-652.

Honys, D., and Twell, D. (2004). Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. Genome Biol. **5:** R85.

Hosp, J., Tashpulatov, A., Roessner, U., Barsova, E., Katholnigg, H., Steinborn, R., Melikant, B., Lukyanov, S., Heberle-Bors, E., and Touraev, A. (2007). Transcriptional and metabolic profiles of stress-induced, embryogenic tobacco microspores. Plant Mol. Biol. **63:** 137-149.

Ilić-Grubor, K., Attree, S.M., and Fowke, L.C. (1998). Comparative morphological study of zygotic and microspore-derived embryos of *Brassica napus* L. as revealed by scanning electron microscopy. Ann. Bot. **82:** 157-165.

Indrianto, A., Barinova, I., Touraev, A., and Heberle-Bors, E. (2001). Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. Planta **212:** 163-174.

Islam, S., and Tuteja, N. (2012). Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Sci. **182:** 134-144.

Joosen, R., Cordewener, J., Supena, E.D.J., Vorst, O., Lammers, M., Maliepaard, C., Zeilmaker, T., Miki, B., America, T., and Custers, J. (2007). Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol. **144:** 155-172.

Kaltchuk-Santos, E., Mariath, J.E., Mundstock, E., Hu, C.-y., and Bodanese-Zanettini, M.H. (1997). Cytological analysis of early microspore divisions and embryo formation in cultured soybean anthers. Plant Cell Tiss. Org. Cult. **49:** 107-115.

Kaplan, D.R., and Cooke, T.J. (1997). Fundamental concepts in the embryogenesis of dicotyledons: a morphological interpretation of embryo mutants. Plant Cell **9:** 1903-1919.

Kasha, K., Hu, T., Oro, R., Simion, E., and Shim, Y. (2001). Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. J. Exp. Bot. **52:** 1227-1238.

Kim, M., Kim, J., Yoon, M., Choi, D.-I., and Lee, K.-M. (2004). Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). Plant Cell Tiss. Org. Cult. **77:** 63-72.

Kumari, M., Clarke, H.J., Small, I., and Siddique, K.H. (2009). Albinism in plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. Crit. Rev. Plant Sci. **28:** 393-409.

Loraine, A., McCormick, S., Estrada, A., Patel, K., and Qin, P. (2013). High-throughput sequencing of Arabidopsis thaliana pollen cDNA uncovers novel transcription and alternative splicing. Plant Physiol.

Lotan, T., Ohto, M.-a., Yee, K.M., West, M.A., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1998). *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell **93:** 1195-1205.

Magnard, J.-L., Le Deunff, E., Domenech, J., Rogowsky, P.M., Testillano, P.S., Rougier, M., Risueño, M.C., Vergne, P., and Dumas, C. (2000). Genes normally expressed in the endosperm are expressed at early stages of microspore embryogenesis in maize. Plant Mol. Biol. **44:** 559-574.

Maheshwari, P. (1950). An Introduction to the Embryology of Angiosperms. ((New York: McGraw-Hill)).

Malik, M.R., Wang, F., Dirpaul, J.M., Zhou, N., Polowick, P.L., Ferrie, A.M., and Krochko, J.E. (2007). Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol. **144:** 134-154.

Maluszynski, M., Kasha, K.J., Forster, B.P., and Szarejko, I. (2003). Doubled haploid production in crop plants: a manual. (Dordrecht: Kluwer Academic Publishers).

Mansfield, S., and Briarty, L. (1991). Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can. J. Botany **69:** 461-476.

Mansfield, S., Briarty, L., and Erni, S. (1991). Early embryogenesis in *Arabidopsis thaliana*. I. The mature embryo sac. Can. J. Botany **69:** 447-460.

Maraschin, S.F., de Priester, W., Spaink, H.P., and Wang, M. (2005a). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. J. Exp. Bot. **56:** 1711-1726.

Maraschin, S.F., Lamers, G.E., de Pater, B.S., Spaink, H.P., and Wang, M. (2003). 14-3-3 isoforms and pattern formation during barley microspore embryogenesis. J. Exp. Bot. **54:** 1033-1043.

Maraschin, S.F., Vennik, M., Lamers, G.E., Spaink, H.P., and Wang, M. (2005b). Time-lapse tracking of barley androgenesis reveals position-determined cell death within pro-embryos. Planta **220:** 531-540.

Maraschin, S.F., Caspers, M., Potokina, E., Wülfert, F., Graner, A., Spaink, H.P., and Wang, M. (2006). cDNA array analysis of stress-induced gene expression in barley androgenesis. Physiol. Plant. **127:** 535-550.

Maraschin, S.F., Gaussand, G., Pulido, A., Olmedilla, A., Lamers, G.E., Korthout, H., Spaink, H.P., and Wang, M. (2005c). Programmed cell death during the transition from multicellular structures to globular embryos in barley androgenesis. Planta **221:** 459-470.

Mascarenhas, J.P. (1990). Gene activity during pollen development. Annu. Rev. Plant Biol. **41:** 317-338.

Mascarenhas, J.P. (1993). Molecular mechanisms of pollen tube growth and differentiation. Plant Cell **5:** 1303-1314.

Massonneau, A., Coronado, M.-J., Audran, A., Bagniewska, A., Mòl, R., Testillano, P.S., Goralski, G., Dumas, C., Risueño, M.-C., and Matthys-Rochon, E. (2005). Multicellular structures developing during maize microspore culture express endosperm and embryo-specific genes and show different embryogenic potentialities. Eur. J. Cell Biol. **84:** 663-675.

Mayer, U., Ruiz, R.A.T., Berleth, T., Miseéra, S., and Juürgens, G. (1991). Mutations affecting body organization in the *Arabidopsis* embryo. Nature **353:** 402-407.

McCormick, S. (1993). Male gametophyte development. Plant Cell **5:** 1265-1275.

Mordhorst, A.P., Toonen, M.A., de Vries, S.C., and Meinke, D. (1997). Plant embryogenesis. Crit. Rev. Plant Sci. **16:** 535-576.

Muñoz-Amatriaín, M., Svensson, J.T., Castillo, A.-M., Cistué, L., Close, T.J., and Vallés, M.-P. (2006). Transcriptome analysis of barley anthers: effect of mannitol treatment on microspore embryogenesis. Physiol. Plant. **127:** 551-560.

Nitta, T., Takahata, Y., and Kaizuma, N. (1997). Scanning electron microscopy of microspore embryogenesis in *Brassica* spp. Plant Cell Rep. **16:** 406-410.

Olsen, F.L. (1987). Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carlsberg Res. Commun **52:** 393-404.

Prem, D., Solís, M.-T., Bárány, I., Rodríguez-Sanz, H., Risueño, M.C., and Testillano, P.S. (2012). A new microspore embryogenesis system under low temperature which mimics zygotic embryogenesis initials, expresses auxin and efficiently regenerates doubled-haploid plants in Brassica napus. BMC Plant Biol. **12:** 127.

Pulido, A., Bakos, F., Devic, M., Barnabás, B., and Olmedilla, A. (2009). HvPG1 and ECA1: two genes activated transcriptionally in the transition of barley microspores from the gametophytic to the embryogenic pathway. Plant Cell Rep. **28:** 551-559.

Pulido, A., Bakos, F., Castillo, A., Vallés, M., Barnabas, B., and Olmedilla, A. (2005). Cytological and ultrastructural changes induced in anther and isolated-microspore cultures in barley: Fe deposits in isolated-microspore cultures. J. Struct. Biol. **149:** 170-181.

Raghavan, V. (1986). Embryogenesis in Angiosperms. A Developmental and Experimental Study. (Cambridge, London: Cambridge University Press).

Ramesar-Fortner, N.S., and Yeung, E.C. (2006). Physiological influences in the development and function of the shoot apical meristem of microspore-derived embryos of *Brassica napus*' Topas'. Botany **84:** 371-383.

Reichheld, J.-P., Vernoux, T., Lardon, F., Van Montagu, M., and Inzé, D. (1999). Specific checkpoints regulate plant cell cycle progression in response to oxidative stress. Plant J. **17:** 647-656.

Reynolds, T.L. (1993). A cytological analysis of microspores of *Triticum aestivum* (Poaceae) during normal ontogeny and induced embryogenic development. Am. J. Bot. **80:** 569-576.

Reynolds, T.L. (1997). Pollen embryogenesis. Plant Mol. Biol. **33:** 1-10.

Rybczynski, J., Simonson, R., and Baenziger, P. (1991). Evidence for microspore embryogenesis in wheat anther culture. In Vitro Cellular & Developmental Biology-Plant **27:** 168-174.

Sánchez-Díaz, R.A., Castillo, A.M., and Vallés, M.P. (2013). Microspore embryogenesis in wheat: new marker genes for early, middle and late stages of embryo development. Plant reproduction **26:** 287-296.

Sabelli, P.A. (2012). Seed development: A comparative overview on biology of morphology, physiology, and biochemistry between monocot and dicot plants. In Seed Development: OMICS Technologies toward Improvement of Seed Quality and Crop Yield, G.K. Agrawal and R. Rakwal, eds (Dordrecht: Springer), pp. 3-25.

Salas, P., Rivas-Sendra, A., Prohens, J., and Seguí-Simarro, J.M. (2012). Influence of the stage for anther excision and heterostyly in embryogenesis induction from eggplant anther cultures. Euphytica **184:** 235-250.

Schrick, K., Mayer, U., Horrichs, A., Kuhnt, C., Bellini, C., Dangl, J., Schmidt, J., and Jürgens, G. (2000). FACKEL is a sterol C-14 reductase required for organized cell division and expansion in Arabidopsis embryogenesis. Genes Dev. **14:** 1471-1484.

Schulze, D., and Pauls, K. (2002). Flow cytometric analysis of cellulose tracks development of embryogenic Brassica cells in microspore cultures. New Phytol. **154:** 249-254.

Seguí-Simarro, J.M. (2010). Androgenesis revisited. Bot. Rev. **76:** 377-404.

Seguí-Simarro, J.M., Corral-Martínez, P., Parra-Vega, V., and González-García, B. (2011). Androgenesis in recalcitrant solanaceous crops. Plant Cell Rep. **30:** 765-778.

Shariatpanahi, M.E., Bal, U., Heberle-Bors, E., and Touraev, A. (2006a). Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiol. Plant. **127:** 519-534.

Shariatpanahi, M.E., Belogradova, K., Hessamvaziri, L., Heberle-Bors, E., and Touraev, A. (2006b). Efficient embryogenesis and regeneration in freshly isolated and cultured wheat (*Triticum aestivum* L.) microspores without stress pretreatment. Plant Cell Rep. **25:** 1294-1299.

Sheridan, W.F., and Clark, J.K. (1993). Mutational analysis of morphogenesis of the maize embryo. Plant J. **3:** 347-358.

Simmonds, D.H., and Keller, W.A. (1999). Significance of preprophase bands of microtubules in the induction of microspore embryogenesis of *Brassica napus*. Planta **208:** 383-391.

Solís, M.-T., Pintos, B., Prado, M.-J., Bueno, M.-Á., Raska, I., Risueño, M.-C., and Testillano, P.S. (2008). Early markers of *in vitro* microspore reprogramming to embryogenesis in olive (*Olea europaea* L.). Plant Sci. **174:** 597-605.

Soriano, M., Cistué, L., and Castillo, A. (2008). Enhanced induction of microspore embryogenesis after n-butanol treatment in wheat (*Triticum aestivum* L.) anther culture. Plant Cell Rep. **27:** 805-811.

Stasolla, C., Belmonte, M.F., Tahir, M., Elhiti, M., Khamiss, K., Joosen, R., Maliepaard, C., Sharpe, A., Gjetvaj, B., and Boutilier, K. (2008). Buthionine sulfoximine (BSO)-mediated improvement in cultured embryo quality in vitro entails changes in ascorbate metabolism, meristem development and embryo maturation. Planta **228:** 255-272.

Stirn, S., Mordhorst, A.P., Fuchs, S., and Lörz, H. (1995). Molecular and biochemical markers for embryogenic potential and regenerative capacity of barley (*Hordeum vulgare* L.) cell cultures. Plant Sci. **106:** 195-206.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2001). *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. USA **98:** 11806-11811.

Straatman, K., and Schel, J. (2001). Distribution of splicing proteins and putative coiled bodies during pollen development and androgenesis in *Brassica napus* L. Protoplasma **216:** 191-200.

Sunderland, N. (1974). Anther culture as a means of haploid induction. In Haploids in Higher Plants: Advances and Potential, K.J. Kasha, ed (Guelph: The University of Guelph.

Sunderland, N., and Wicks, F.M. (1971). Embryoid formation in pollen grains of *Nicotiana tabacum*. J. Exp. Bot. **22:** 213-226.

Sunderland, N., and Evans, L. (1980). Multicellular pollen formation in cultured barley anthers II. The A, B, and C pathways. J. Exp. Bot. **31:** 501-514.

Supena, E.D.J., Winarto, B., Riksen, T., Dubas, E., van Lammeren, A., Offringa, R., Boutilier, K., and Custers, J. (2008). Regeneration of zygotic-like microspore-derived embryos suggests an important role for the suspensor in early embryo patterning. J. Exp. Bot. **59:** 803-814.

Szakács, É., and Barnabás, B. (1988). Cytological aspects of in vitro androgenesis in wheat (*Triticum aestivum* L.) using fluorescent microscopy. Sex. Plant Reprod. **1:** 217-222.

Szakács, É., and Barnabás, B. (1995). The effect of colchicine treatment on microspore division and microspore-derived embryo differentiation in wheat (*Triticum aestivum* L.) anther culture. Euphytica **83:** 209-213.

Tanaka, I., and Ito, M. (1981). Control of division patterns in explanted microspores of *Tulipa gesneriana*. Protoplasma **108:** 329-340.

Tang, X., Liu, Y., He, Y., Ma, L., and Sun, M.-x. (2013). Exine dehiscing induces rape microspore polarity, which results in different daughter cell fate and fixes the apical-basal axis of the embryo. J. Exp. Bot. **64:** 215-228.

Telmer, C.A., Newcomb, W., and Simmonds, D.H. (1993). Microspore development in *Brassica napus* and the effect of high temperature on division in vivo and in vitro. Protoplasma **172:** 154-165.

Telmer, C.A., Newcomb, W., and Simmonds, D.H. (1995). Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma **185:** 106-112.

Testillano, P.S., Ramírez, C., Domenech, J., Coronado, M.-J., Vergne, P., Matthys-Rochon, E., and Risueño, M.C. (2002). Young microspore-derived maize embryos show two domains with defined features also present in zygotic embryogenesis. Int. J. Dev. Biol. **46:** 1035-1048.

Thakur, P., Kumar, S., Malik, J.A., Berger, J.D., and Nayyar, H. (2010). Cold stress effects on reproductive development in grain crops: an overview. Environ. Exp. Bot. **67:** 429-443.

Torp, A., and Andersen, S. (2009). Albinism in microspore culture. In Advances in Haploid Production in Higher Plants, A. Touraev, B.P. Forster, and S.M. Jain, eds (Dordrecht: Springer), pp. 155-160.

Torres-Ruiz, R.A., and Jurgens, G. (1994). Mutations in the *FASS* gene uncouple pattern formation and morphogenesis in *Arabidopsis* development. Development **120:** 2967-2978.

Touraev, A., Lezin, F., Heberle-Bors, E., and Vicente, O. (1995). Maintenance of gametophytic development after symmetrical division in tobacco microspore culture. Sex. Plant Reprod. **8:** 70-76.

Tran, F., Penniket, C., Patel, R.V., Provart, N.J., Laroche, A., Rowland, O., and Robert, L.S. (2013). Developmental transcriptional profiling reveals key insights into Triticeae reproductive development. Plant J.

Tsuwamoto, R., Fukuoka, H., and Takahata, Y. (2007). Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. Planta **225:** 641-652.

Twell, D., Park, S.K., and Lalanne, E. (1998). Asymmetric division and cell-fate determination in developing pollen. Trends Plant Sci. **3:** 305-310.

Ueda, M., Zhang, Z., and Laux, T. (2011). Transcriptional activation of *Arabidopsis* axis patterning genes *WOX8/9* links zygote polarity to embryo development. Dev. Cell **20:** 264-270.

Whittle, C.A., Malik, M.R., Li, R., and Krochko, J.E. (2010). Comparative transcript analyses of the ovule, microspore, and mature pollen in *Brassica napus*. Plant Mol. Biol. **72:** 279-299.

Yeung, E.C. (2002). The canola microspore-derived embryo as a model system to study developmental processes in plants. J. Plant Biol **45:** 119-133.

Yeung, E.C., and Meinke, D.W. (1993). Embryogenesis in angiosperms: development of the suspensor. Plant Cell **5:** 1371-1381.

Yeung, E.C., Rahman, M.H., and Thorpe, T.A. (1996). Comparative development of zygotic and microspore-derived embryos in *Brassica napus* L. CV Topas. I. Histodifferentiation. Int. J. Plant Sci. **157:** 27-39.

Ylstra, B., and McCormick, S. (1999). Analysis of mRNA stabilities during pollen development and in BY2 cells. Plant J. **20:** 101-108.

Zaki, M., and Dickinson, H. (1990). Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma **156:** 149-162.

Zaki, M., and Dickinson, H. (1991). Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Plant Reprod. **4:** 48-55.

Zhang, Z., and Laux, T. (2011). The asymmetric division of the *Arabidopsis* zygote: from cell polarity to an embryo axis. Sex. Plant Reprod. **24:** 161-169.

Zhao, J.-P., Simmonds, D.H., and Newcomb, W. (1996). Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. Planta **198:** 433-439.

Zimmerman, J.L. (1993). Somatic embryogenesis: a model for early development in higher plants. Plant Cell **5:** 1411-1423.

Żur, I., Dubas, E., Słomka, A., Dubert, F., Kuta, E., and Płażek, A. (2013). Failure of androgenesis in Miscanthus× giganteus in vitro culture of cytologically unbalanced microspores. Plant reproduction **26:** 297-307.

Chapter 3

The Histone Deacetylase Inhibitor Trichostatin A Promotes Totipotency in the Male Gametophyte

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Abstract

The haploid male gametophyte, the pollen grain, is a terminally differentiated structure whose function ends at fertilization. Plant breeding and propagation widely use haploid embryo production from *in vitro* cultured male gametophytes, but this technique remains poorly understood at the mechanistic level. Here, we show that histone deacetylases (HDACs) regulate the switch to haploid embryogenesis. Blocking HDAC activity with trichostatin A (TSA) in cultured male gametophytes of *Brassica napus* leads to a large increase in the proportion of cells that switch from pollen to embryogenic growth. Embryogenic growth is enhanced by, but not dependent on the high temperature stress that is normally used to induce haploid embryogenesis in *B. napus*. The male gametophyte of *Arabidopsis thaliana,* which is recalcitrant to haploid embryo development in culture, also forms embryogenic cell clusters after TSA treatment. Genetic analysis suggests that the HDAC protein HDA17 plays a role in this process. TSA treatment of male gametophytes is associated with the hyperacetylation of histones H3 and H4. We propose that the totipotency of the male gametophyte is kept in check by an HDAC-dependent mechanism, and that the stress treatments used to induce haploid embryo development in culture impinge on this HDAC-dependent pathway.

Introduction

Many plant cells have the inherent ability to regenerate a complete organism from single cells or tissues, a process referred to as totipotency. During sexual reproduction, cellular totipotency is restricted to the zygote, which is formed in the seed from fusion of the egg and sperm cell upon fertilisation. Sustained division of the zygote generates the embryo, which contains the basic body plan of the adult plant. Establishment of groups of pluripotent stem cells in the stem cell niche of the embryonic shoot and root apical meristems ensures the continuous post-embryonic growth and development of new lateral organs that is characteristic of plant development (Bennett and Scheres, 2010; Besnard et al., 2011). Embryo development also occurs in the absence of egg cell fertilisation during apomixis, a type of asexual seed development. Totipotency in apomictic plants is restricted to the gametophytic and sporophytic cells that normally contribute to the development of the seed and its precursors, including the unfertilised egg cell and surrounding sporophytic tissues (Bicknell and Koltunow, 2004).

The totipotency of plant cells reaches its highest expression in tissue culture. The ability of a cell to undergo embryogenesis *in vitro* is both an inherent and an acquired characteristic that requires the right combination of explant and culture environment. A wide variety of cells have the potential to develop into embryos, including haploid gametophytic cells, such as the cells of pollen and embryo sacs (Forster et al., 2007; Seguí-Simarro, 2010), as well as somatic cells derived from all three tissue layers of the plant (Gaj, 2004; Rose et al., 2010). The treatments used to induce embryogenesis are diverse and range from application of exogenous growth regulators to abiotic stress. Under the appropriate conditions, the explant resumes cell division and produces histodifferentiated embryos, either directly from the explant or indirectly from a callus. The morphological and cellular changes that occur during *in vitro* embryogenesis have been described in some species (Raghavan, 2004; Seguí-Simarro and Nuez, 2008), but there is still very little known about the initial steps involved in the acquisition and expression of totipotency in individual cells, and many of the assumed diagnostic features of cultured embryogenic cells are being revised in the light of live imaging studies (Daghma et al., 2012; Tang et al., 2013). Molecular screens have been performed to identify the changes that occur during *in vitro* embryogenesis; however, the range of species, explants and culture conditions that have been used, combined with low percentage of cells that form embryos, has made it difficult to develop a unified concept of the totipotent plant cell.

In *Arabidopsis thaliana*, dynamic regulation of gene expression at the chromatin level plays a major role in translating the developmental and environmental signals that regulate cell totipotency *in planta* (Zhang and Ogas, 2009). The basic structural and functional unit of chromatin is the nucleosome, which comprises DNA wrapped around a histone octamer, and associated linker histones (Andrews and Luger, 2011). Nucleosomes can present a physical barrier restricting the access of non-histone proteins to DNA due to the strong interaction between the positively charged histones and negatively charged DNA. Transcription requires physical binding of transcription factors to open DNA; thus, controlling the compaction and accessibility of loci through nucleosomes offers a dynamic means to control gene expression. Dynamic changes in chromatin structure and gene transcription are mediated primarily by the interwoven processes of chromatin remodelling and histone modification (Jiang and Pugh, 2009; Henikoff and Shilatifard, 2011). Chromatin remodelling proteins use the energy from ATP hydrolysis to remove or reposition nucleosomes (Flaus and Owen-Hughes, 2011),

while histone modifying enzymes chemically modify lysines and other amino acids on the exposed N-terminal tails of histones to change their charge and interaction with DNA and other proteins (Bannister and Kouzarides, 2011).

A number of conserved chromatin modifying proteins ensure the successful transition from embryo development to post-embryonic growth by repressing pathways controlling embryo cell proliferation and identity during germination (Zhang and Ogas, 2009). Loss-of-function mutants of these proteins express embryo identity genes ectopically and develop somatic embryos on seedlings. These chromatin modifying proteins include members of the *Arabidopsis* SWI/SNF and CHD classes of chromatin-remodelling ATPases (Ogas et al., 1999), members of the Polycomb Group (PcG) Repressive Complex 1 (PRC1) and 2 (PRC2), which deposit repressive marks on histones, respectively, histone 2A lysine 119 (H2AK119) ubiquitination and histone 3 lysine 27 (H3K27) trimethylation (Chanvivattana et al., 2004; Schubert et al., 2005; Makarevich et al., 2006; Chen et al., 2009; Bratzel et al., 2010; Bouyer et al., 2011; Tang et al., 2012), and histone deacetylases, which create a repressive transcriptional state by removing acetyl groups from the lysines of histone tails (Tanaka et al., 2008). The large number of proteins that play a role in this process, combined with the potential crosstalk between different chromatin modifying proteins (Zhang et al., 2012) ensures a multi-level dynamic control over cell totipotency.

Changes in chromatin organisation and modification are often associated with *in vitro* plant regeneration (Miguel and Marum, 2011), but there are few examples where chromatin level changes are known to play a direct role in this process (He et al., 2012). In this report, we examined the role of chromatin modification in defining the totipotency of haploid embryo cultures derived from *Brassica napus* male gametophytes. The male gametophyte is a highly differentiated structure whose function ends at fertilisation. During male gametophyte development, the single-celled haploid microspore divides to form a multicellular pollen grain, containing a vegetative cell, and two generative (sperm) cells that participate in double fertilisation. This developmental pathway can be disrupted when microspores and pollen are cultured *in vitro* and induced to form haploid embryos. This form of haploid embryogenesis, referred to as microspore embryogenesis, pollen embryogenesis or androgenesis, is induced by exposing anthers or isolated microspores/pollen to abiotic or chemical stress during *in vitro* culture (Touraev et al., 1997). These stress treatments induce sustained, sporophytic division of the microspores/pollen leading to the formation of a

histodifferentiated haploid embryo. The ability of haploid embryos to convert spontaneously, or after treatment with chromosome doubling agents, to doubled-haploid plants is widely exploited as a means to generate homozygous plants in a single generation, and has numerous breeding and trait discovery applications (Touraev et al., 1997; Forster et al., 2007).

Haploid embryogenesis was described almost 50 years ago in *Datura stromonium* (Guha and Maheshwari, 1964). Since then, many cell biological studies in model species such as tobacco, barley and *Brassica*, have laid a solid foundation for understanding the cellular events that accompany haploid embryogenesis, yet the mechanism underlying this change in developmental pathways is still not known. Here we show that chemical inhibition of histone deacetylase (HDAC) activity using trichostatin A (TSA; Finnin et al., 1999) induces massive embryogenic cell proliferation in the male gametophyte of *B. napus*, even in the absence of the heat stress treatment that is normally used to induce haploid embryogenesis. Our results suggest that inhibition of HDAC activity or downstream HDAC-mediated pathways plays a major role in the initiation of stress-induced haploid embryogenesis.

Results

TSA induces hyperproliferation

We determined whether altering the histone acetylation status of cultured microspores and pollen by treating them with the HDAC inhibitor, TSA, would relieve any of the developmental blocks in haploid embryo formation in the poorly-responsive *B. napus* genotype DH12075. *B napus* is one of the most well-studied models for microspore embryogenesis (Custers et al., 2001). Heat-stress treatment is used to induce microspore embryogenesis in this and other *Brassica* species.

We examined the development of *B. napus* microspore cultures by staining heat-stressed (hereafter referred to as control) and heat-stressed plus TSA-treated male gametophytes at different developmental stages with the nuclear dye, DAPI. Initial dosage experiments were used to establish the minimal exposure time (20 h) in relation to the specific phenotypes discussed below (Supplemental Figure 1, Supplemental Dataset 1).

After two days of heat stress, microspores/pollen in control cultures either arrested, continued gametophyte development, or divided sporophytically. Male gametophyte development in culture followed the same course of development as in the anther (Figure

1A-C). The single-celled microspore divided asymmetrically (pollen mitosis I, PM I) to generate a pollen grain with a large vegetative cell containing a diffusely-stained nucleus and a smaller generative cell with a more compact nucleus. The vegetative cell did not divide again, while the generative cell divided once (pollen mitosis II, PM II) to produce the two gametes, the sperm cells. In *Brassica napus*, sporophytic growth initiates in the late uninucleate microspore, and to a lesser extent from the cell-cycle arrested vegetative cell of the early bicellular pollen grain (Sunderland, 1974; Fan et al., 1988). As previously described, microspores that divided sporophytically contained two large, diffusely-stained nuclei, rather than the large vegetative nucleus and small generative nucleus produced after PM I (Figure 1D). Male gametophytes that divided sporophytically after PM I, which was rarely observed (<1%) in control cultures from this genotype, contained a small generative-like cell in addition to the larger sporophytic cells (Figure 1E). After heat stress treatment, the majority of the cells in the control culture were gametophytic-like or had died (Figure 1G; Supplemental Dataset 1), as evidenced by the lack of DAPI staining. Up to 6% of the population divided sporophytically within the first two days of culture, producing cell clusters with two to six nuclei (Figure 1G; Supplemental Dataset 1). Sporophytically-dividing cells were observed in cultures containing pure populations of microspores and in cultures containing a mixture of microspores and binucleate pollen.

The combined effect of heat stress and 0.5 µM TSA on sporophytic cell division after two days of culture was dramatic, with up to 80% of the population dividing sporophytically (Figure 1H; Supplemental Dataset 1). Unlike the control cultures, the largest increase in the proportion of sporophytically-divided structures was observed in cultures that initially contained a mixture of microspores and binucleate pollen. The majority of sporophytically-divided cells in these cultures contained two to six diffusely-stained nuclei, as in control cultures. Unlike control cultures, approximately 10% of the sporophytically-divided cells also contained one or more generative-like nuclei (Figure 1F). The low frequency of cells with generative-like nuclei is surprising considering the 40 to 60% binucleate pollen that was present at the start of culture in some samples. The generative nucleus may degrade, or its chromatin may adopt a less condensed status, similar to that of the vegetative nucleus.

Our observations indicate that TSA-mediated loss of HDAC activity in cultured microspores/pollen induces a high frequency of sporophytic cell division, and suggests that HDAC proteins play a major role in controlling cell cycle progression during male

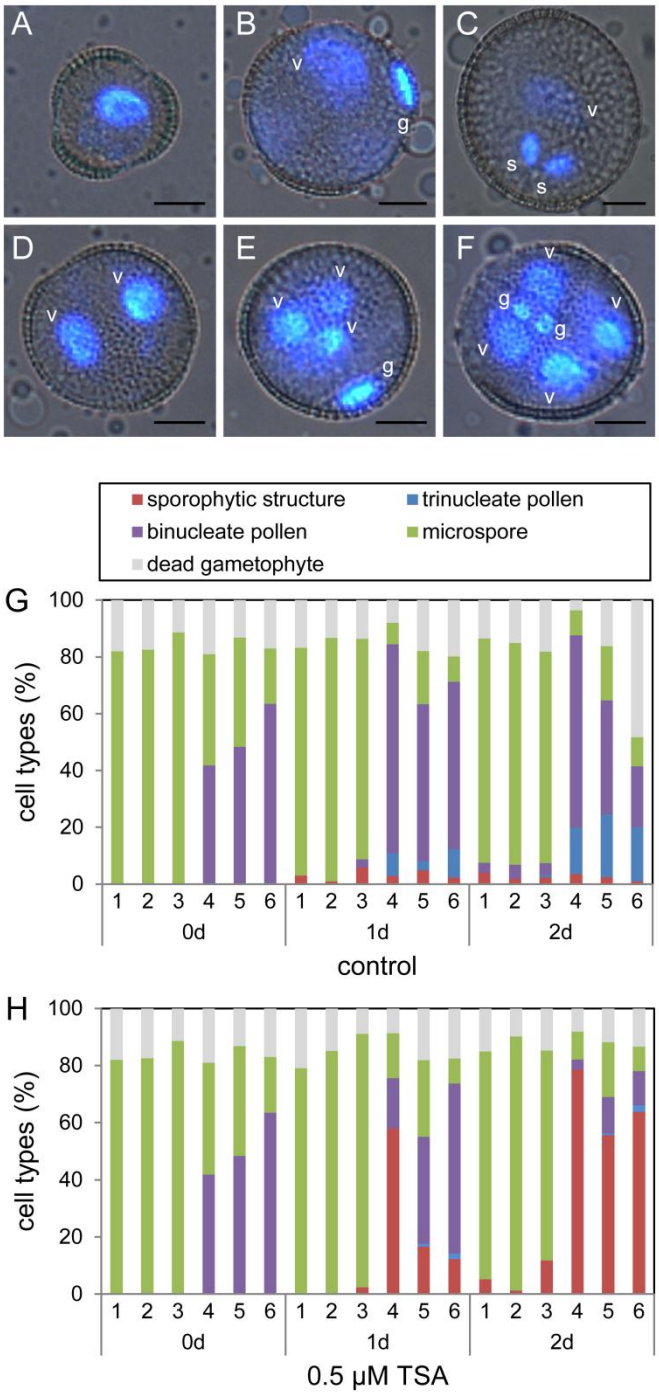

Figure 1. Effect of TSA on early cell division patterns in *B. napus* microspore culture. DAPI-stained gametophytic (**A-C**) and sporophytic structures (**D-F**) present in the first two days of microspore culture. (**A**) Microspore. (**B**) Binucleate pollen. (**C**) Trinucleate pollen. (**D**) Sporophytically-divided cell with two large vegetative-like nuclei. (**E**) Sporophytic structure with three vegetative-like nuclei and one small generative-like nucleus. (**F**) Multinucleate sporophytic structure with four vegetative-like nuclei and two generative-like nuclei. (**G-H**) The percentage of different cell types observed in control (**G**) and TSA-treated cultures (**H**). The developmental stages of the starting cultures (1-8) are ranked from youngest to oldest. The percentages of each structure in control and TSA-treated cultures are shown in Supplemental Dataset 1. v, vegetative(-like) nucleus; g, generative(-like) nucleus; s, sperm nucleus. Scale bar, 10 µm.

gametophyte development. The combined effect of heat-stress and TSA treatment was more potent than that of heat-stress alone, both in terms of the range of developmental stages and the proportion of male gametophytes that were induced to divide sporophytically.

TSA and heat-stress induce similar developmental changes

The developmental fate of heat-stressed control cultures and cultures exposed to both heat-stress and TSA was followed by examining older cultures in more detail. Our initial

experiments showed that the proportion of dividing cells, as well as their developmental fate was influenced by the concentration of TSA that was applied to the culture. We therefore treated heat-stressed microspores and pollen with a range of TSA concentrations and examined the cultures after five and 15 days using DAPI staining to characterize the different multicellular structures that developed.

Four types of sporophytic structures could be distinguished in five-day old control cultures (Figure 2A-E; Supplemental Figure 2; Supplemental Dataset 1), some of which have been previously described in microspore cultures of other *Brassica* genotypes (Fan et al., 1988; Telmer et al., 1995; Ilić-Grubor et al., 1998). Type I structures corresponded to the classical embryo-forming structures that are routinely observed in microspore culture (Fan et al., 1988; Telmer et al., 1995). After five days of culture these multicellular structures contained up to 40 nuclei that were still enclosed in the pollen wall (exine; Figure 2B). Cell walls formed in Type I structures, but were not clearly visible, as described previously (Fan et al., 1988). These embryogenic multicellular structures were only observed in control cultures that initially contained a mixture of late uninucleate microspores and early binucleate pollen, and only comprised a small proportion of the population of dividing cells (0.5%). Type II structures were the most abundant structures present in five-day control cultures. They were callus-like, less compact than Type I structures, and contained up to five cells that had already started to emerge from the exine (Figure 2C; Fan et al., 1988). Type III structures contained two to three large, diffusely DAPI-stained nuclei that were no longer enclosed by the exine. The exine remained attached to these cell clusters and was often associated with a generative-like nucleus (Figure 2D). Type IV structures, which were rarely observed in control cultures, comprised loose callus-like clusters with well-defined cell walls (Figure 2E; Fan et al., 1988; Ilić-Grubor et al., 1998).

The same sporophytic structures were observed in five-day-old cultures that received a combined heat-stress and TSA-treatment, but were found in different proportions depending on the concentration of TSA that was applied (Figure 2A; Supplemental Figure 2; Supplemental Dataset 1). Treatment with heat-stress and TSA mainly induced the formation of Type II structures (up to 77% versus 7% in the control cultures) and Type IV structures (up to 32% versus 0.5% in the control cultures). Type I classical embryogenic structures were observed at a low frequency when 0.5 μM TSA was added to the culture medium (up to 1% versus 0.5% in the control cultures), but were more abundant (up to 3%) when a ten-fold

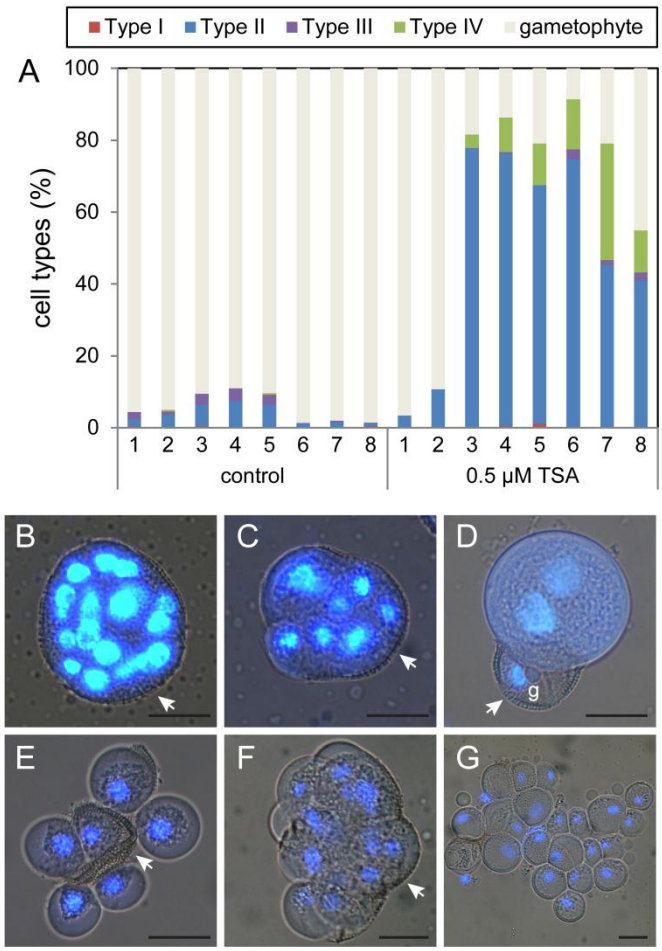

Figure 2. Effect of TSA on sporophytic growth in *B. napus* microspore culture. **(A)** The percentage of cells that formed pollen or divided sporophytically (Type I-IV) after five days of microspore culture. The corresponding structures (Type I-IV) are shown in (**B-E**). The developmental stages of the starting cultures (1-8) are ranked from youngest to oldest. The percentages of each structure in control and TSA-treated cultures are shown in Supplemental Dataset 1 and Supplemental Figure 2A. **(B-G)** Sporophytic structures after five (**B-E**) and 15 (**F-G**) days of culture. **(B)** Type I, classical embryo-forming structure. **(C)** Type II, compact callus-like structure. **(D)** Type III, extruded sporophytic structure. **(E)** Type IV, loose callus-like structure. **(F)** Type II, structure. **(G)** Type IV, structure. Nuclei in (**B-G**) are stained with DAPI. Arrow, intact (**B**) or broken (**C**, **D**, **E, F**) exine. g, generative(-like) nucleus. Scale bar, 20 µm.

lower concentration of TSA was applied.

With the exception of Type III structures, all of the sporophytic multicellular structures observed in control and heat-stress plus TSA-treated cultures were still present and had increased in size after 15 days of culture (Figure 2F, G), and were still more abundant in TSA-treated cultures. Types II and IV cell clusters eventually stopped growing and died in both control and TSA-treated cultures.

Only a small percentage of the heat-stressed microspores/pollen normally develop into differentiated embryos (Supplemental Figure 2C; Supplemental Dataset 1). Compared to control cultures, treatment of heat-stressed cultures with 0.05 µM TSA increased the total embryo yield by increasing the range of developmental stages that produced histodifferentiated embryos, as well as the embryo production per stage. Treatment with higher concentrations of TSA had a negative effect on embryo yield. These data indicate that

TSA not only has a positive effect on the formation of embryogenic cells, but that it also enhances the formation of histodifferentiated embryos.

We determined whether the heat-stress treatment used to induce haploid embryogenesis is required for the TSA cell proliferation phenotype. Microspore cultures incubated at temperatures lower than 33 ^{o}C divide sporophytically, with the proportion of dividing cells depending on the culture temperature and stage of male gametophyte development, but produce fewer or no embryos compared to 33^{o}C cultures. We observed an increase in the percentage of sporophytic divisions when TSA was applied to microspore cultures growing at either 18 or 25 ^{o}C, as well as a corresponding increase in embryo production at 25 ^{o}C (Supplemental Figures 3 and 4; Supplemental Dataset 1). Up to 0.2% embryo production was observed in TSA-treated cultures compared to practically no embryo production in the non TSA-treated controls (Supplemental Figure 3C). Higher TSA concentrations were needed to induce cell proliferation and embryo production at these lower temperatures compared to cultures grown at 33 ^{o}C.

Together, our data indicate that treatment with TSA and heat-stress mediate similar developmental changes in microspore culture, and suggest that the heat stress treatment used to induce haploid embryogenesis impinges on pathways that are controlled by HDAC proteins.

Sporophytic cell clusters are embryogenic

The cell clusters that formed in heat-stressed, TSA-treated cultures resembled those found in control cultures that are only exposed to a heat-stress treatment. They included classical embryogenic structures, as well as structures that have been classified as non-embryogenic based on their unorganized structure, early release from the exine, and the lack of a protoderm, which is considered a hallmark for commitment to embryo development in culture (Fan et al., 1988; Telmer et al., 1995; Ilić-Grubor et al., 1998). We used RT-PCR and GFP reporter lines to determine whether the different types of sporophytic structures that develop in control- and TSA-treated cultures are embryogenic.

The expression of four embryo-expressed transcription factors genes, *BABY BOOM* (*BBM*; Boutilier et al., 2002), *LEAFY COTYLEDON1* (*LEC1*; Lotan et al., 1998), *LEC2* Stone et al., 2001) and *FUSCA3* (To et al., 2006) is positively correlated with the embryogenic potential of *B. napus* microspore cultures (Malik et al., 2007). Our RT-PCR analysis showed that expression

of these four genes was enhanced when microspore cultures were treated with TSA, regardless of the culture temperature (Supplemental Figure 5), suggesting that TSA treatment is sufficient to activate embryo gene expression in microspore culture.

We developed *B. napus* GFP reporter lines for two *Arabidopsis* embryo-expressed genes, *LEC1* (*LEC1:LEC1-GFP*) and *GLYCINE-RICH PROTEIN* (*GRP, GRP:GFP-GUS*), to identify the specific structures that contribute to the enhanced embryo gene expression observed in TSA-treated cultures. The early embryo expression of both GFP reporters was confirmed in *B. napus* zygotic embryos, where *LEC1* expression was detected as early as the 2-cell stage and *GRP* expression from the zygote stage onward (Supplemental Figure 6). Neither gene was expressed during the uni-, bi- or trinucleate stages of male gametophyte development in the anther (Supplemental Figure 7).

We used the predominately nuclear localisation of the LEC1-GFP fusion to more precisely follow the developmental identity of the different cell types found in microspore cultures within the first three days of culture (Figure 3; Supplemental Table 1). In control (heat-stressed) microspore cultures, LEC1-GFP was expressed in microspore-like structures, and in cells that contained two large, diffusely stained nuclei, but not in bi- or trinucleate pollen-like structures (Figure 3A, C, E, G). After TSA treatment of heat-stressed microspores, LEC1-GFP was also observed in the same structures as in the control cultures, but also in bi- and trinucleate pollen-like structures (Figure 3B, D, F, H). In pollen-like structures or sporophytically-divided cells with a generative-like nucleus, LEC1-GFP was expressed in both the vegetative- and generative-like nuclei, but never in generative-like nuclei alone (Figure 3D, F, I).

Later, in both control and TSA-treated cultures, *LEC1* and *GRP* expression was observed in the classical embryo (Type I) structures, in the same spatial pattern as in zygotic embryos (Figure 4A, B; Supplemental Figure 6; Supplemental Table 1), as well as throughout the Type II and IV sporophytic structures (Figure 4C, D, G, H). However, only *LEC1* expression was detected in Type III structures (Figure 4E, F). An overview of the *LEC1* and *GRP* expression patterns in control and TSA-treated cultures is shown in Supplemental Table 1. The data suggest that TSA-treated and control microspore cultures show similar developmental changes. Surprisingly, microspores/pollen can be reprogrammed to embryo development following heat-stress/TSA treatment in the absence of cell division. Simultaneous exposure to TSA and exposure to TSA and heat-stress appears to have a stronger effect than

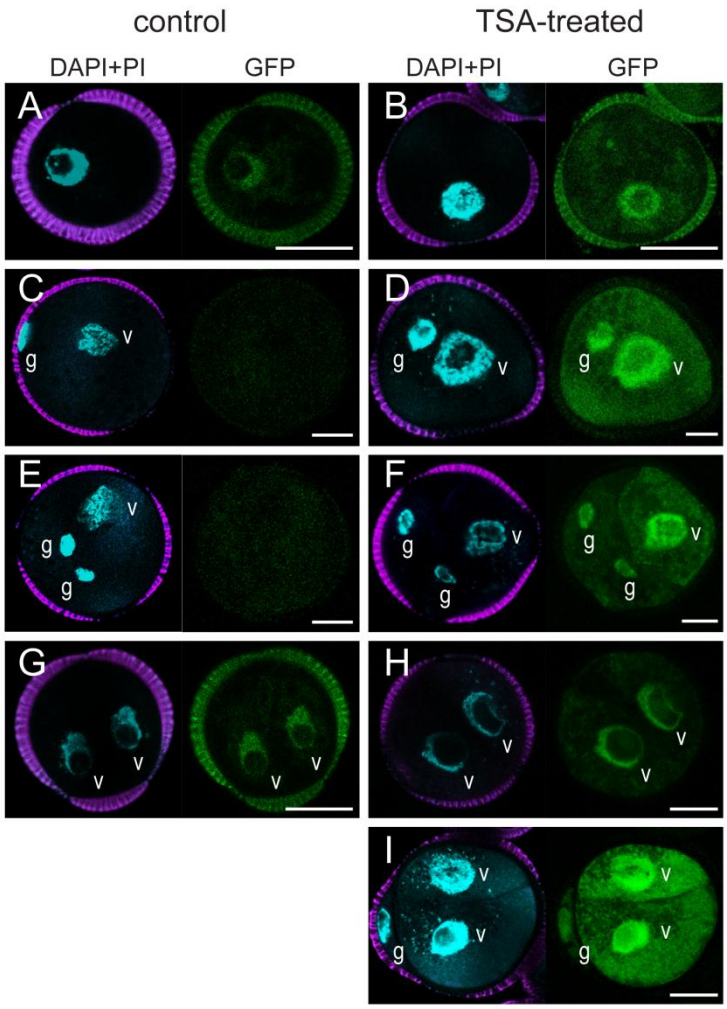

Figure 3. TSA enhances embryo marker expression in *B. napus* microspore culture.

Expression of *LEC1:LEC1-GFP* in two day-old control **(A, C, E, G)** and TSA-treated **(B, D, F, H, I)** cultures. **(A, B)** Microspore-like structure. **(C, D)** Binucleate pollen-like structure. **(E, F)** Trinucleate pollen-like structure. **(G, H, I)** Sporophytically-divided structures derived from division of a microspore (**G**, **H**) and a binucleate pollen (**I**). For each panel, the image on the left side of each panel shows the combined fluorescence from PI (magenta) and DAPI staining (blue) and the image on the right side, the GFP fluorescence (green). The green exine in (**A**), (**B**) and (**G**) is due to autofluorescence.g, generative-like nucleus; v, vegetative-like nucleus. Scale bar, 10 µm.

heat-stress alone, in that the embryo gene expression is activated in both vegetative- and generative-like cells.

TSA induces totipotency in *Arabidopsis* male gametophytes

The production of haploid callus and embryos from cultured anthers has been described for a number of *Arabidopsis* ecotypes and species (Gresshoff and Doy, 1972; Scholl and Amos, 1980), but we and others have not been able to reproduce these protocols, nor have we been able to develop an isolated microspore culture system for *Arabidopsis*. Nonetheless, we were able to induce multicellular structures that resemble the Type II and IV structures seen in *Brassica* microspore culture when stage 11 *Arabidopsis* anthers were cultured at 25 ^{o}C with 0.5 µM TSA (Figure 5A, B). Growth of donor plants at a low temperature and *in vitro* culture at a higher temperature, as in *B. napus* (Custers, 2003), was not necessary, nor did it improve the production of sporophytic structures. The percentage of male gametophytes

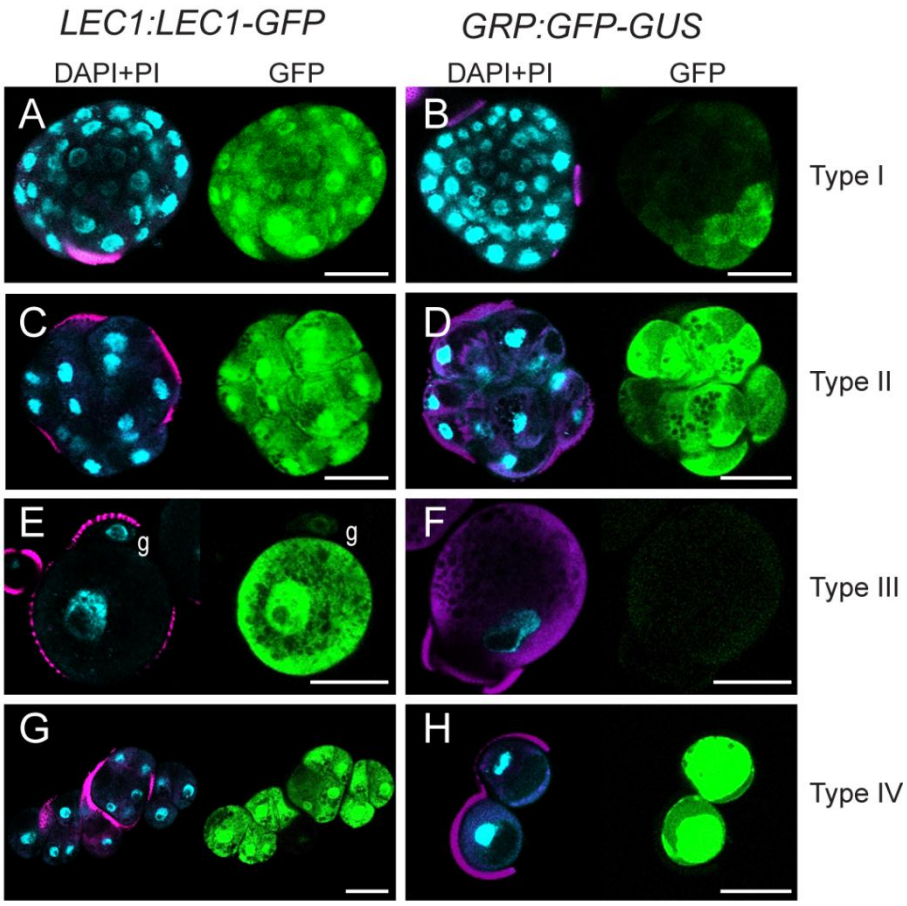

Figure 4. Embryo marker expression in sporophytic structures.

(A, C, E, G) *LEC1:LEC1-GFP* and **(B, D, F, H)** *GRP:GFP-GUS* expression in five to eight day-old TSA-treated microspore cultures. The same patterns of expression were observed in control cultures. **(A**, **B)** Type I structures. **(C**, **D)** Type II compact callus-like structures. **(E**, **F)** Type III extruded sporophytic structures. g, generative-like nucleus. **(G**, **H)** Type IV loose callus-like structure. For each panel, the image on the left side of each panel shows the combined fluorescence from PI- (magenta) and DAPI staining (blue) and the image on the right side, the GFP fluorescence (green). Scale bar, 25 µm.

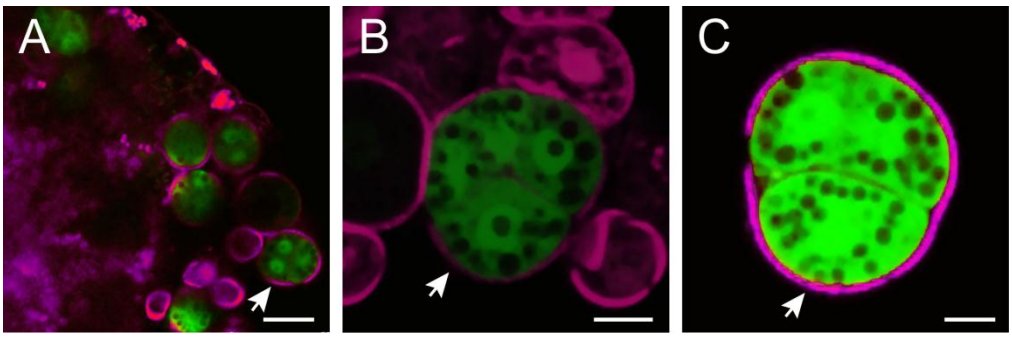

Figure 5. TSA induces embryogenic cell divisions in *Arabidopsis* male gametophytes.

(A, **B)** Expression of *LEC1:LEC1-GFP* and **(C)** *ENODL4:GFP* in a Type II compact callus-like structure in a TSA-treated anther. The exine (arrow) still surrounds the sporophytic structures. GFP, green; PI, magenta. All images are from five day-old anther cultures. Scale bar, 25 µm (A) and 10 µm (B, C).

that divided sporophytically in TSA-treated Col-0 anthers was consistent across experiments (ca. 4%; Supplemental Dataset 1), provided the anthers were carefully staged, whereas sporophytic divisions were never observed in anthers cultured without TSA (Figure 5C). We examined expression of the *LEC1* and *GRP* marker lines in TSA treated cultures, but could only detect *LEC1* expression (Figure 5D). However, a third embryo reporter, *EARLY NODULIN-LIKE PROTEIN 4:GFP (ENODL4:GFP;* Supplemental Figure 4), was expressed in the TSA-induced multicellular structures (Figure 5E). Together, these data demonstrate that TSA also induces embryogenic growth in *Arabidopsis* male gametophytes but is not sufficient to induce the formation of histodifferentiated embryos.

Behaviour of *hda* and *rbr* mutants in *Arabidopsis* anther culture

We determined whether T-DNA insertions in *Arabidopsis* HDAC genes phenocopy TSA-treated anthers. *Arabidopsis* contains 18 HDAC genes (referred to as *HDA1-18*) grouped into the Rpd3/Hda1, HD-tuin and sirtuin families (Hollender and Liu, 2008). TSA targets Zn^{2+}-dependent HDACs (Grozinger and Schreiber, 2002; Gregoretti et al., 2004), which correspond to the Rpd3/HDA1 and HD-tuin type HDACs (Hollender and Liu, 2008). We examined lines with T-DNA insertions in the genes encoding Rpd3/HDA1 and HD-tuin- type HDAs (Supplemental Table 2) for ectopic divisions of the male gametophyte during normal anther development *in situ,* but did not observe any changes in the pollen cell division pattern in these lines. Likewise, none of the *hda* insertion lines showed sporophytic divisions in cultured pollen in the absence of TSA. It was difficult to evaluate TSA hypersensitive responses for some of the *hda* T-DNA insertion mutants; for example *hda6* and *hda8*, due to their variable response in culture, however, among the mutants that showed more consistent responses, one mutant *hda17* showed a small, but significant increase in the percentage of sporophytic cell divisions relative to the control (Figure 6A; Supplemental Dataset 1). This data suggest that the activity of at least one HDAC, HDA17, is required to suppress ectopic cell divisions in *Arabidopsis* pollen.

The mammalian Retinoblastoma protein (pRB) recruits HDAC1 to repress cell cycle gene transcription (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). In maize, the Rb protein RETINOBLASTOMA-RELATED1 (RBR1) interacts with an Rpd3-type histone deacetylase, Rpd3I, and together these proteins repress gene transcription (Rossi et al., 2003). In *Arabidopsis*, loss of RBR function leads to hyperproliferation of the male and female gametophyte (Ebel

et al., 2004; Chen et al., 2009). Given the similarities between the *rbr* phenotype and TSA treatment, and the interaction of retinoblastoma proteins with HDACs, we examined whether RBR plays a role in TSA-mediated cell totipotency. Homozygous *rbr* mutants are gametophytic lethal; therefore the experiments were performed on heterozygous *rbr-3* anthers (*rbr-3/+),* a reported null allele (Ebel et al., 2004), which contain 50% *rbr* pollen. We scored the developing structures as dead, gametophytic, *rbr*-like or Type II, TSA-like. The *rbr* phenotype is most penetrant during the bicellular stage of pollen development and is characterized by structures with multiple vegetative cells, and to a lesser extent, extra generative-like cells (Figure 6B; Chen et al., 2009). The TSA phenotype differed from the *rbr* phenotype in that the TSA-like cells were larger and contained more vegetative-like cells than *rbr* cells, and were surrounded by a stretched or broken exine (Figure 6C). If an RBR-HDAC interaction is required to prevent sporophytic cell divisions in culture, then culturing *rbr* mutant pollen without TSA could induce TSA-like divisions. Culture of *rbr-3* anthers with TSA should not have an additive effect on the percentage of sporophytic divisions, except when TSA inhibition of HDAC activity is incomplete. We observed ectopic cell proliferation of male gametophytes when *rbr-3/+* anthers were cultured in the absence of TSA. The typical compact *rbr*-like structures with up to 6 nuclei that develop *in planta* were observed, but at a lower frequency than was reported (Figure 6D; Supplemental Dataset 1; Chen et al., 2009). Strikingly, *rbr-3/+* anthers cultured in the absence of TSA also produced a low percentage (0.5%) of enlarged and loosely-connected Type II multicellular structures (Figure 6D), which we have never observed in cultured control anthers from wild-type plants. We did not observe any differences between TSA-treated wild-type and TSA-treated *rbr-3/+* anthers, other than the typical *rbr*-like divisions that are observed in the *rbr-3* line; however, compared to untreated *rbr-3/+* anthers, TSA-treated *rbr-3/+* anthers showed a decrease in the frequency of *rbr*-like divisions. Together, our experiments with cultured *rbr-3/+* anthers suggest that loss of RBR function is sufficient to induce the formation of embryogenic cell clusters in *Arabidopsis* anther culture in the absence of TSA. The decrease in the frequency of *rbr*-like divisions after TSA treatment may reflect a requirement for HDAC activity in promoting the typical *rbr*-type cell-cycle progression.

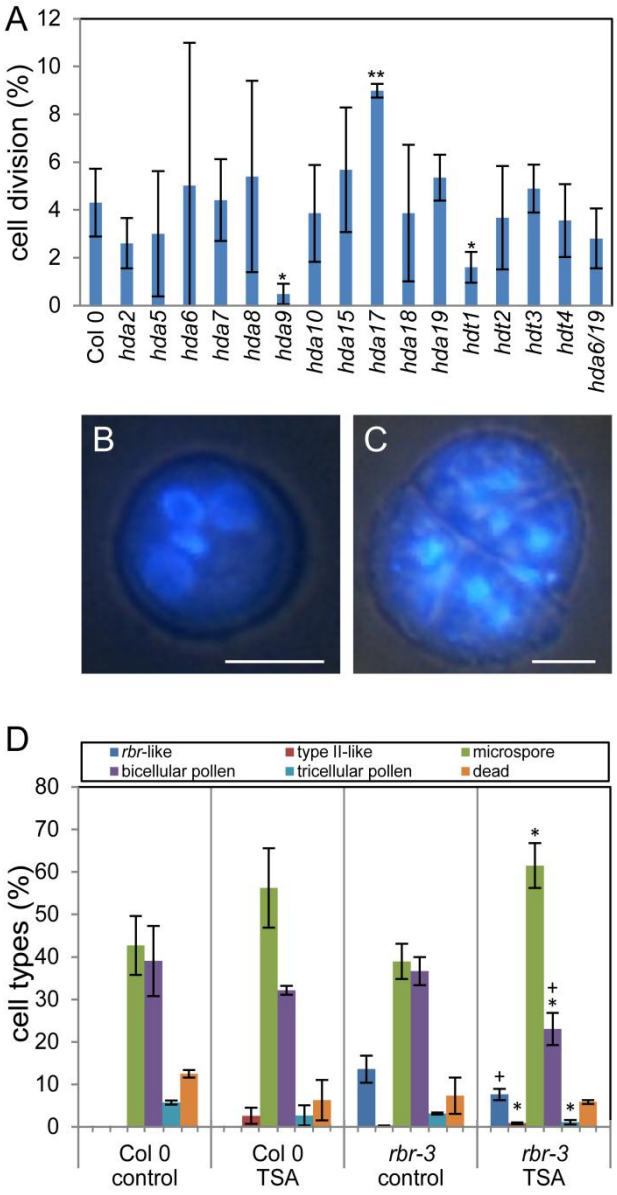

Figure 6. Behaviour of *hda* and *rbr* mutants in *Arabidopsis* anther culture. **(A)** Sporophytic cell division in male gametophytes from *hda* T-DNA insertion lines treated with 0.5 µM TSA. Statistical comparison (Student's *t*-test) was made between the TSA-treated Col-0 anthers and the TSA-treated *hda* mutant anthers. *p <0.05; **p<0.01. **(B, C)** Multicellular sporophytic structures observed in cultured *rbr-3/+* anthers. **(B)** *rbr*-like multicellular structure with three vegetative-like cells and one generative-like cell. Scale bar, 10 µm. **(C)** Type II multicellular structure with eight nuclei. **(D)** Relative proportion of the different cell types observed in *rbr-3/+* anther cultures treated with 0.5 µM TSA or DMSO (control cultures). Statistically significant differences were observed between the response of TSA treated and untreated *rbr-3* anthers (⋆, p<0.05; Student's *t*-test) and TSA treated *rbr-3* and Col-0 anthers (+, p<0.05; Student's *t*-test). Samples were observed five days after the start of culture. The percentages of each structure from Col-0 and mutants in control and TSA-treated cultures are shown in Supplemental Dataset 1.

TSA promotes histone acetylation

HDACs deacetylate the lysine residues of both histone and non-histone proteins (Xu et al., 2007). We used an acetylated lysine antibody in combination with protein gel blotting to identify proteins whose acetylation status changes in 8 hour heat-stressed, TSA-treated *B. napus* microspore cultures compared to heat-stressed control cultures. We observed increased protein acetylation in low molecular mass proteins in the range of 10 to 25 kDa in the TSA treated cultures compared to control cultures (Figure 7A). As these proteins are in the size range of histones (Moehs et al., 1988), we examined the acetylation status of the

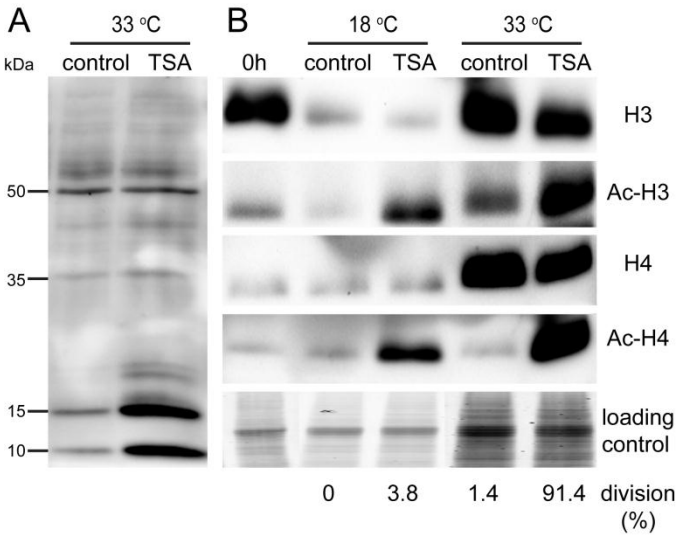

Figure 7. TSA enhances histone acetylation. **(A)** Immunoblot analysis of total acetylated proteins in microspore cultures treated for eight hours with DMSO (control) or TSA. Proteins in the range of 10-25 kDa are differentially acetylated after TSA treatment compared to the control. **(B)** Immunoblot of total and acetylated (Ac) histone H3 and H4 in microspore cultures treated for eight hours with DMSO (control) or TSA. The percentages of sporophytic divisions in the different cultures at day 5 are shown under each sample.

most commonly acetylated histones, histones H3 and H4 (Loidl, 2004), during microspore culture using acetylated histone H3 (Ac-H3) and H4 (Ac-H4) antibodies. Microspore cultures were started from buds containing mostly binucleate pollen and placed for eight hours at either 18 ^{o}C or 33 ^{o}C with or without 0.5 µM TSA. As expected, TSA greatly enhanced sporophytic divisions at 18 ^{o}C and 33 ^{o}C compared to the untreated controls (Figure 7B). Although this increase in cell division had no clear effect on the total amount of histone H3 and H4 detected in the control and TSA-treated cultures, the level of histone H3 and H4 acetylation increased dramatically in the TSA-treated cultures relative to control cultures, both at 18 ^{o}C and at 33 ^{o}C (Figure 7B). Our data suggest that the main effect of decreased HDAC activity following TSA treatment in microspore culture is the increased acetylation of histones.

TSA induces changes in cell wall, auxin, and cell division pathways

The acetylation status of histones generally correlates with the transcriptional competence of the associated locus, with highly acetylated and deacetylated histones associated with permissive and repressive gene expression states, respectively. We used microarray analysis to identify the early gene expression changes in *B. napus* microspore cultures that are associated with TSA treatment. Freshly isolated microspore cultures were heat-stressed to induce embryogenesis and at the same time treated for eight hours with TSA, either alone, or together with the protein translation inhibitor cycloheximide to identify

primary transcriptional changes. Only a small number of statistically significantly up or down-regulated genes were identified (407; Supplemental Figure 8A), and at most a four-fold change in gene expression was observed between the two treatments and their respective controls (Supplemental Dataset 2). Nonetheless, the differential regulation of a selection of these probes could be confirmed independently by quantitative real-time RT-PCR, although the observed fold changes were much larger than in the microarray analysis (Supplemental Figure 8B).

We observed down-regulation of a small number of genes (51; Supplemental Figure 6A; Supplemental Dataset 2), more than half of which are pollen- or pollen-tube expressed genes (Supplemental Figure 9). Despite these changes, the expression of the majority of the highly abundant, late pollen transcripts was not affected (Supplemental Dataset 2). In contrast to the down-regulated gene set, the set of genes that were significantly upregulated after TSA treatment are associated with a wide range of developmental stages and functions (Supplemental Dataset 2). We observed an increase of *LEC1* expression after TSA treatment, but this was not accompanied by major changes in expression of other early embryo genes or embryo identity regulators (Supplemental Dataset 2). Thus, a large up-regulation of embryo gene expression appears to occur later, after one to two days of culture, when expression of the GFP-based embryo reporters is first observed.

Although short inhibition of HDAC activity is not associated with major transcriptional changes of embryo or pollen-identity genes, we were able to identify a number of specific pathways that were altered after microspores were treated for eight hours with TSA (Supplemental Dataset 2). One notable category of upregulated genes includes genes involved in cell wall loosening and degradation (xyloglucan endotransglucosylase/hydrolases, *XTH*), as well as pectin depolymerisation and solubilisation (polygalacturonases, pectin polygalacturonase beta-subunit protein, pectin methylesterase, pectin esterases and pectate lyases), and cellulose hydrolysis (*CELLULASE 1* (*CEL1*) and *CEL2*). A number of auxin-related genes are also upregulated after TSA treatment (Supplemental Dataset 2). These include two *GH3* genes (*GH3.1* and *DFL1*/*GH3.6*) that in Arabidopsis are known to increase the pool of inactive amino-acid conjugated IAA (Staswick et al., 2005) and that are induced by auxin and stress, as well as *ILR1,* which is involved in increasing free auxin levels through cleavage of indole-3-acetic acid (IAA)-amino acid conjugates (Rampey et al., 2004). Genes involved in auxin transport through efflux (*PIN1, PIN3, PIN7*; Friml et al., 2002) and influx (*AUX1;* Yang et

al., 2006), and in auxin signalling (*AFB3;* Dharmasiri et al., 2005), as well as auxin upregulated genes of unknown function (*AIR12;* Preger et al., 2009) were also upregulated after TSA treatment. A small number of cell-cycle-related genes are also upregulated after TSA treatment. One of the early genes that is upregulated by TSA encodes an E2Fd/DEL2 transcription factor, and the more downstream gene targets include two positive regulators of the (G1-to-S phase) of the cell cycle, *CYCLIN D3;3* (*CYCD3;3*) and a *CYCLIN D1*-like gene.

Together these results indicate that TSA treatment within the first few hours of microspore culture alters the expression of a diverse, but limited set of genes. These data are consistent with studies in mammalian cells where only a small proportion of genes responded to HDAC inhibition (Halsall et al., 2012).

Discussion

Here we show that inhibition of histone deacetylase activity is sufficient to induce embryogenic growth in cultured pollen of *B. napus* and *Arabidopsis*. Many different stressors are used to induce haploid embryogenesis in plants (Islam and Tuteja, 2012); thus, in this respect, the deregulation of HDACs or HDAC-mediated pathways by stress and the accompanying changes in histone acetylation status, could provide a single, common regulation point for the induction of haploid embryogenesis.

Competence for haploid embryogenesis

The developmental stage of the vegetative cell plays a major role in its responsiveness to heat stress and TSA. In the majority of species, the stress treatment is most effective in triggering sustained cell division in microspore culture shortly before or after PM I (Touraev et al., 1997). Unlike heat stress, TSA, alone or in combination with heat-stress, is highly effective at later stages of pollen development, and has a much stronger effect than heat-stress with respect to the proportion of cells that divide sporophytically. TSA may be a more potent inducer of sporophytic growth due to its ability to more completely inhibit individual HDACs or to inhibit a wider range of HDAC-mediated processes than heat-stress alone. In line with this, a relatively high concentration of TSA in combination with heat stress enhances divisions that mainly result in disorganized embryogenic structures, but a relatively low concentration of TSA in combination with heat-stress more closely mimics the effect of heat-stress alone, enhancing the formation of both histodifferentiated embryos and non-

viable disorganized embryogenic structures. Culture at lower temperatures dampens the effect of TSA, such that fewer cells divide, and a higher concentration of TSA is needed to induce embryo and embryogenic cell formation at 18 ^{o}C than at 33 ^{o}C. In a similar fashion, a more severe, 41 ^{o}C heat-stress is required to induce sporophytic divisions and embryogenesis in *B. napus* pollen at the late bicellular stage (Binarova et al., 1997). Together these data suggest that HDACs (directly or indirectly) mediate the inhibition of cell cycle progression that is gradually imposed on the vegetative cell, and that release of this inhibition is required for embryogenic growth in culture.

Role of cell cycle progression in haploid embryo induction

The CYCD/RB pathway is an evolutionarily conserved control point in the progression through the G1 phase of the cell cycle (Gutzat et al., 2012). One group of major players are the E2F transcription factors, which dimerize with DP proteins to activate transcription of genes that facilitate the G1/S transition and S-phase. E2F proteins are inhibited through binding to Rb (Harbour and Dean, 2000), and Rb negatively affects transcription through its interaction with HDACs and other chromatin modification proteins (Zhang et al., 2000). Phosphorylation of Rb by a complex of CYCD proteins and associated kinases releases Rb from E2F, allowing expression of genes for DNA replication and passage through G1/S (Dewitte and Murray, 2003). In plants, altered expression of different components of the G1/S phase of the cell-cycle leads to changes in cell proliferation, in the length of the cell cycle, and in the amount of endoreduplication (reviewed in Gutierrez, 2009).

Our microarray analysis showed that TSA treatment induced the expression of genes associated with G1/S cell cycle progression. One of these genes, *E2Fd/DEL2* encodes one of three atypical *Arabidopsis* E2Fs that do not bind to the DP or Rb proteins due to the lack of a DP-dimerisation domain and an Rb binding pocket (Lammens et al., 2009). Sozzani et al. (2010) have shown that DEL2 promotes cell proliferation in *Arabidopsis* roots. The expression of two CYCD-encoding genes, *CYCD3;3* and *CYCLIN D1-like*, were also upregulated after TSA treatment. CYCD proteins play important roles in integrating nutritional and hormone signals with the cell cycle response in tissue culture (Riou-Khamlichi et al., 1999, 2000). In *Arabidopsis*, *CYCD1;1* is expressed early during seed germination, where it is rate-limiting for cell-cycle progression in the root meristem (Masubelele et al., 2005), while CYCD3;3 together with CYCD3;1 and CYCD3;2 maintains the mitotic cycle in roots, preventing

endoreduplication (Dewitte et al., 2007). These results suggest that HDAC inhibition induces cell proliferation through activation of components of the G1-to-S phase transition, and that this involves both retinoblastoma-dependent and independent pathways.

We also examined whether the *Arabidopsis rbr* mutant, the only plant cell cycle-related mutant that shows ectopic cell proliferation during male gametophyte development (Johnston et al., 2008; Chen et al., 2009), also plays a role in TSA-mediated haploid embryogenesis. During anther development, *rbr* pollen shows limited ectopic division of the vegetative cell, and to a lesser extent, the generative cell of bicellular pollen. Analysis of microspore and pollen cell fate markers indicates that the cell fate change from the microspore-to vegetative cell identity is delayed in *rbr* pollen, and that changes in cell fate are a secondary consequence of the change in cell division pattern (Chen et al., 2009). The *rbr* phenotype is therefore different from that observed after application of heat-stress or heat-stress plus TSA, where changes in cell fate and cell division appear to be uncoupled. This observation, combined with the low frequency of Type II embryogenic cell clusters found in cultured *rbr-3/+* anthers, as well as the activation genes involved in both RBR-dependent and independent pathways by TSA suggest that RBR plays a role in repressing totipotent growth in anther culture, but is not a major regulator of this pathway.

Acquisition of embryo identity

The progression of haploid development requires re-activation of cell division in the vegetative cell; however, our examination of embryo reporter lines and microarray analysis showed that embryo gene expression activated prior to cell division. The observation is striking, as establishment of new cell fates in both plants and animals usually requires an asymmetric cell division (reviewed in De Smet and Beeckman, 2011) or formation of transit amplifying (meristem) cells (reviewed in Sablowski, 2011).

The expression of embryo identity genes prior to sporophytic division raises the question whether their expression is sufficient to drive cell division toward totipotent growth or whether additional factors are required to mediate this change in development. Ectopic expression of Arabidopsis transcription factors such as BABY BOOM (Boutilier et al., 2002) and the *LEC1* CCAAT-box binding factor examined in this study, is sufficient to induce *de novo* formation of somatic embryos on seedlings (Lotan et al., 1998; Stone et al., 2001; Yang and Zhang, 2010). However, not all tissues form somatic embryos in response to

overexpression of these proteins, suggesting that so-called 'competence factors' are also required to promote this change in cell fate. In microspore culture, this competence might be provided by the combination of developmental stage, culture medium and induction treatment.

Our microarray analysis suggested that the massive embryogenic cell proliferation induced by TSA is not accompanied by a rapid decrease in pollen gene expression. Pollen transcripts have been observed in *B. napus* microspore culture for up to five days after the start of culture, and have also been observed in purified embryogenic structures (Joosen et al., 2007; Malik et al., 2007). It is not clear whether the persistence of pollen transcripts in microspore culture reflects their inherent abundance or stability, or the active maintenance of pollen identity in both gametophytic and embryogenic structures (Joosen et al., 2007; Malik et al., 2007). It will be interesting to determine whether the co-expression of pollen and embryo gene expression programs affects the subsequent development of haploid embryo formation.

The most common route to sporophytic growth in *B. napus* and other species is through ectopic division of the microspore or vegetative cell of binucleate pollen (Sunderland and Wicks, 1971; Fan et al., 1988; Indrianto et al., 2001; Pulido et al., 2005). Sporophytic structures composed of generative-like and vegetative-like nuclei can be observed occasionally (Fan et al., 1988; Reynolds, 1993; González-Melendi et al., 1996; Kaltchuk-Santos et al., 1997; González and Jouve, 2005), but it is not known whether sustained division of generative-like cells contributes to the formation of viable embryos. Our results show that the *LEC1* embryo reporters are expressed only in the microspore and vegetative cell after heat-stress treatment, while exposure to heat-stress and TSA also induces *LEC1* expression in the generative cell. The fate of these 'embryogenic' generative-like nuclei is not clear, as we did not observe generative-like nuclei in multinucleate sporophytic structures. One highly speculative possibility that needs further investigation is that the chromatin of the generative nucleus decondenses, assuming a structure similar to that of the vegetative cell, and then undergoes sustained division, either alone or together with the vegetative-like nuclei. Alternatively, the generative cell and/or its derivatives could simply degenerate and not form part of the embryo (Corral-Martínez et al., 2013).

Our analysis of cell fate markers showed that both heat-stressed and heat-stress plus TSA-treated cultures show a high frequency of cell types that express embryo markers, but

that fail to form histodifferentiated embryos. These structures are characterized by clusters of loosely connected cells that are released prematurely from the exine. During successful microspore embryo development, the increase in pressure from the growing cells causes the exine to break after approximately five to six days of culture. Exine rupture is followed by protoderm formation and the establishment of the apical embryo pole at the site of exine rupture and the basal embryo pole away from the site of rupture (Hause et al., 1994; Telmer et al., 1995). In the loosest embryogenic structures (Types III and IV), the cells burst out of the exine as early as the two-celled stage, while more compact structures (Type II) show signs of exine rupture around the 10-cell stage. The reason for premature rupture in these structures is not known. Increased internal pressure from more rapidly expanding cells or loss of exine integrity may stimulate rupture. Cells of type II-IV structures are much larger than the compact structures that form histodifferentiated embryos, but it is not clear whether this increased size causes exine rupture or whether cell expansion occurs after rupture, for example in response to osmotic potential of the medium. The plant cell wall plays an important role in coordinating cellular differentiation, as mutants with defects in cell wall composition or cell adhesion have been shown to undergo unrestricted cell proliferation and callus formation (Frank et al., 2002; Iwai et al., 2002; Krupková et al., 2007; Krupková and Schmülling, 2009). We observed that TSA treatment is associated with an increase in expression of genes encoding cell wall mobilisation enzymes, particularly those involved in the mobilisation of cellulose and pectin. One possibility is that the composition of the cell wall or the connection between cell walls in Type II-IV structures are altered, preventing the proper cell-cell communication required for histodifferentiation.

We also observed an increase in expression of genes involved in the auxin pathway. The role of endogenous auxin and auxin signalling in haploid embryo induction has not been examined, but exogenous auxin is not required to induce microspore embryogenesis in *B. napus*. In contrast, auxin treatment is used routinely to induce embryogenesis from somatic plant tissues (Thomas and Jiménez, 2006). In Arabidopsis, *de novo* auxin biosynthesis, mediated by *YUCCA* gene expression is implicated in somatic embryo induction (Stone et al., 2008; Wójcikowska et al., 2013). We observed increased expression of genes involved in removal auxin from the cell through transport (*PIN*) or conjugation (*GH3*), but also in auxin accumulation through influx (*AFB3*) and deconjugation (*ILR1*). Further research is required to determine whether altered auxin accumulation, as well as altered cell wall composition, is

associated with the induction of callus-like structures or compact, histodifferentiated embryos.

HDA17 inhibits cell proliferation in pollen

Analysis of *hda* T-DNA insertion lines in *Arabidopsis* anther culture suggests that HDA17, an Rpd3-like HDAC, plays a role in suppressing sporophytic growth in anther culture. *hda17* gametophytes showed enhanced sporophytic cell divisions in anther culture, but only in the presence of TSA, suggesting that embryogenic growth requires inhibition of one or more HDAC proteins in addition to HDA17. HDA17 has an incomplete C-terminal deacetylase domain that lacks the conserved active site. TSA binds to the zinc-containing active site of HDACs (Finnin et al., 1999), thus it is unlikely that TSA directly inhibits HDA17 activity, although the deacetylase activity of HDA17 still needs to be demonstrated. The MEF2-interacting transcription repressor (MITR) is a splice variant of HDAC9 that lacks the HDAC domain. MITR represses transcription repression *in trans* by recruiting several different HDACs, and/or a transcriptional corepressor (Zhang et al., 2001). In analogy, the TSA sensitivity of HDA17 may be supplied *in trans* through formation of HDAC protein dimers (Luo et al., 2012) between HDA17 and one or more *Arabidopsis* HDAC proteins.

In *Arabidopsis* seedlings, TSA treatment induces post-germination growth arrest that is accompanied by prolonged expression of embryogenesis-related genes, and the formation of somatic embryo tissue (Tanaka et al., 2008). An *hda6* T-DNA insertion line showed the same growth arrest phenotype when grown in the presence of a much lower concentration of TSA (Tanaka et al., 2008). The residual requirement of TSA for the secondary somatic embryogenesis phenotype in the *hda6* mutant is due to the redundant action of HDA6 and HDA19. Based on their mutant phenotypes, HDA6 and HDA19 could be considered good candidates for TSA-mediated inactivation in microspore culture, but in our hands, neither the single *hda6* or *hda19* mutants, nor the double *hda6*/*hda19* mutants showed enhanced sporophytic division in anther culture, either in the absence or presence of TSA. This suggests that different HDACs and developmental pathways repress embryogenic cell proliferation in microspores/pollen and zygotic embryos. Functional redundancy among *Arabidopsis* HDA proteins is well-documented, thus identification of the HDAC complex that restricts cell proliferation in the developing male gametophyte will require both a systematic screen of higher order *hda* mutant combinations and biochemical analysis.

Methods

Plant material and culture

Brassica napus L. cv. Topas DH4079 and DH12075 were used as donor plants for microspore embryo culture. The *B. napus* plant growth and microspore isolation procedures were performed as described previously (Custers, 2003). Flower buds for microspore culture were grouped by size (measured from the tip of the flower bud to the bottom of the sepal), ranging from 3.0 to 3.5 mm for DH4079 and from 2.6 to 4.0 mm for DH12075. The microspores were isolated and cultured in NLN-13 medium (Lichter, 1982). For induction of embryogenesis, microspores were cultured in the dark at 33 ^{o}C for 20 hours, and subsequently transferred to 25 ^{o}C. Non-induced microspore cultures were cultured continuously at 25 ^{o}C or 18 ^{o}C. Trichostatin A (TSA, Sigma-Aldrich) was prepared in DMSO. Freshly isolated microspores were inoculated in medium containing TSA or the same volume of DMSO as a control, and cultured for 20 hours at the temperature indicated for each experiment. After this period the cultures were centrifuged at 200 g for 3 min, resuspended in fresh NLN-13 medium without TSA, and transferred to 25 ^{o}C.

Arabidopsis thaliana flower buds at stage 11 were collected for anther culture. Flower buds were surface sterilized in 2% bleach for 10 minutes, then rinsed three times in distilled water. The anthers (without filament) were placed in liquid NLN-13 medium containing 0.5 µM TSA or the same volume of DMSO, and then cut in half transversely in the medium to release the microspores. The cultures were placed at 25 ^{o}C for 20 hours in the dark. The medium was then replaced by fresh NLN-13 medium by pipetting gently, and the cultures incubated at 25 ^{o}C for an additional four days. Free and loosely attached microspores were collected and stained with DAPI. *Arabidopsis hda* T-DNA insertion lines were obtained from Nottingham Arabidopsis Stock Centre. At least 300 microspores per sample were counted.

Reporter lines

GFP-based reporter lines were generated for the Arabidopsis embryo-expressed genes, *LEC1* (At1g21970; *LEC1:LEC1-GFP*) and *GRP* (At2g30560; *GRP:GFP-GUS*) and *B. napus ENODL4* (AB836663; *ENODL4:GFP*). For the *LEC1:LEC1-GFP* translational fusion, a 3110 bp DNA fragment comprising 1292 bp upstream of the translational start site and the entire coding region was amplified by PCR and recombined into pGKGWG using the Gateway cloning system (Invitrogen) according to the manufacturer's instructions. *Arabidopsis GRP* encodes

an EGG APPARATUS1-LIKE (EAL) protein (Gray-Mitsumune and Matton, 2006) and is highly similar to a *B. napus* glycine-/proline-rich gene isolated from embryogenic microspore cultures (probe 563; Joosen et al., 2007). The *Arabidopsis GRP:GFP-GUS* transcriptional fusion was made by PCR amplifying a fragment comprising 861 bp upstream of the start codon and Gateway recombination into pBGWFS7,0. The *ENODL4* was identified as an early embryogenesis-expressed gene from *B. napus* microspore culture (Japanese patent No. 35935650). A 1035 bp fragment of the promoter of *ENODL4* (GenBank accession no. AB098076) was cloned by inverse PCR, ligated to the 5'-end of an sGFP: nos terminator fragment and inserted into pBinKH, which is a modified version of a binary vector pGPTV-KAN (Becker et al., 1992). The reporter constructs were transformed to *Agrobacterium tumefaciens* strain C58C1 carrying the pMP90 Ti plasmid and then to *B. napus* DH12075 (Moloney et al., 1989) and/or *Arabidopsis* Col-0 (Clough and Bent, 1998).

Microscopy

The developmental stage and identity of cells in microspore and anther culture were visualized with the nuclear stain 4', 6-diamidino-2-phenylindole (DAPI, 1.25 μg/ml) according to Custers (2003) using a Zeiss Axioskop epifluorescence microscope with filter set no. 02. Approximately two hundred microspores or multicellular clusters were counted for each sample. Confocal laser scanning microscopy (CLSM) was carried out on a Leica DM5500 Q microscope. The GFP was excited with an argon laser line at 488 nm and detected with a 505–530 nm emission filter. Samples were counterstained with DAPI and/or propidium iodide (10 mg/ml; Sigma-Aldrich). Propidium iodide and red autofluorescence were excited at 532 nm and detected with a 620-660 nm emission filter. DAPI was excited at 405 nm and detected with a 440-500 nm emission filter. The optical slices were median filtered with Leica LAS AF software. *Arabidopsis* anthers were cleared in HCG solution (water: chloral hydrate: glycerol; 3:8:1) for 10min, then observed under DIC microscopy with a Nikon OPTIPHOT microscope.

Molecular analyses

Total RNA isolation and on-column DNase digestion were performed using the InviTrap Spin Plant RNA Mini Kit (Invitek) according to the manufacturer's instructions. For RT-PCR, 250 ng of total RNA was used for first-strand cDNA synthesis with the Taqman Reverse

Transcription Reagents Kit (Applied Biosystems). The cycling parameters were: one cycle at 98 ^{o}C for 30 s, 30 cycles comprising 98 ^{o}C for 5 s, 60 ^{o}C for 30 s, followed by 72 ^{o}C for 1 min. The primer sequences are described in Supplemental Table 3. The RT-PCR primers are from Malik et al. (2007). The quantitative RT-PCR primers for microarray validation were designed based on oligonucleotide probes from Affymetrix GeneChip Brassica Exon 1.0ST Array (Malik et al., 2007; Love et al., 2010). The *Arabidopsis hda* T-DNA insertion lines were genotyped using the PCR primers shown in Supplemental Table 2.

Microspore cultures for microarray analysis were cultured at 33 ^{o}C for eight hours with either TSA or TSA plus cycloheximide (CHX, Sigma-Aldrich), both dissolved in DMSO. DMSO or cycloheximide were used as mock treatments for respectively, the TSA and TSA+CHX treatments. The samples were harvested by centrifugation for total RNA was isolation, as described above. One microgram of total RNA from each sample was sent to the NASC Affymetrix Service (http://affymetrix.arabidopsis.info/) for hybridisation to the Affymetrix Brassica Exon 1.0 ST GeneChip. Probe annotations were downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The identifier for the annotation is GPL10733. The expression data was subjected to normalization using the RMA method from the '*affy*' Bioconductor package. Log2-transformed expression values were identified as differentially expressed using a Student's *t*-test. Multiple hypothesis testing correction was done using the Holm's method (Holm, 1979) implemented in the *multtest*'s Bioconductor package. Mapman (Thimm et al., 2004) was used to identify functional categories of differentially-expressed genes. The microarray data has been deposited to the Gene Expression Omnibus (GEO) database (GSE49070).

Immunochemistry

Freshly isolated microspores and microspores cultured for 8 hours under different experimental conditions were harvested by centrifugation. Proteins were extracted by boiling in SDS-sample buffer (30 µl per ml of culture) and electrophoresed in a Midget 12.5% SDS-PAGE gel under reducing conditions. After transfer of the proteins to PVDF membrane and blocking with 5% milk powder in PBS, 0.1% Tween 20, the blots were incubated for 2 hours with primary antibody (1:2000 dilution). The primary antibodies used in this study are as follows: anti-acetyl-Lysine (ICP0380; ImmuneChem Pharmaceuticals), anti-Histone H3 (ab1791; Abcam), anti-Histone H4 (clone 62-141-13; Millipore), and anti-acetyl-Histone H3

and anti-acetyl-Histone H4 (Millipore). Secondary goat anti-rabbit-HRP antibody (Sigma) was used in a 1:2000 dilution and signals were detected by using enhanced chemiluminescence (SuperSignal West Femto Chemiluminescent Substrate, Pierce).

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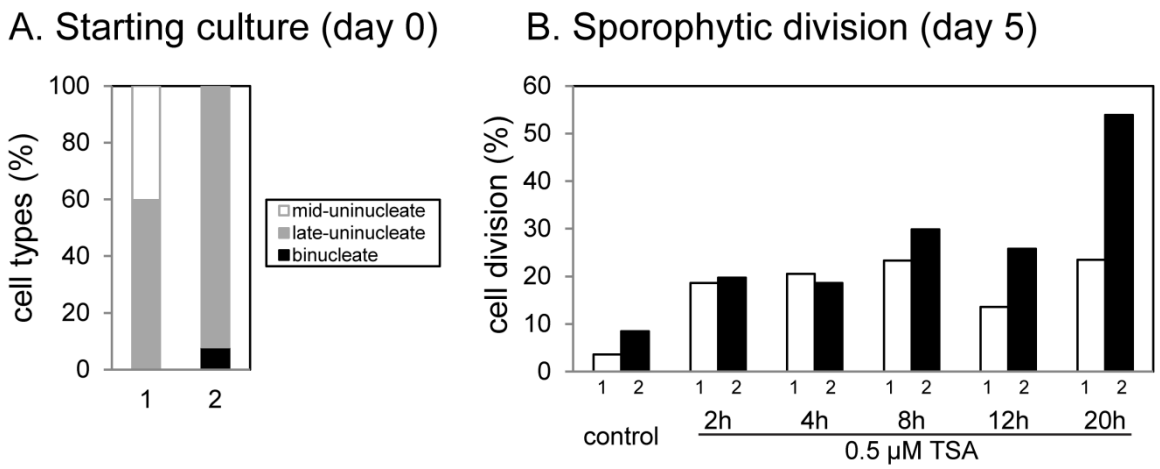
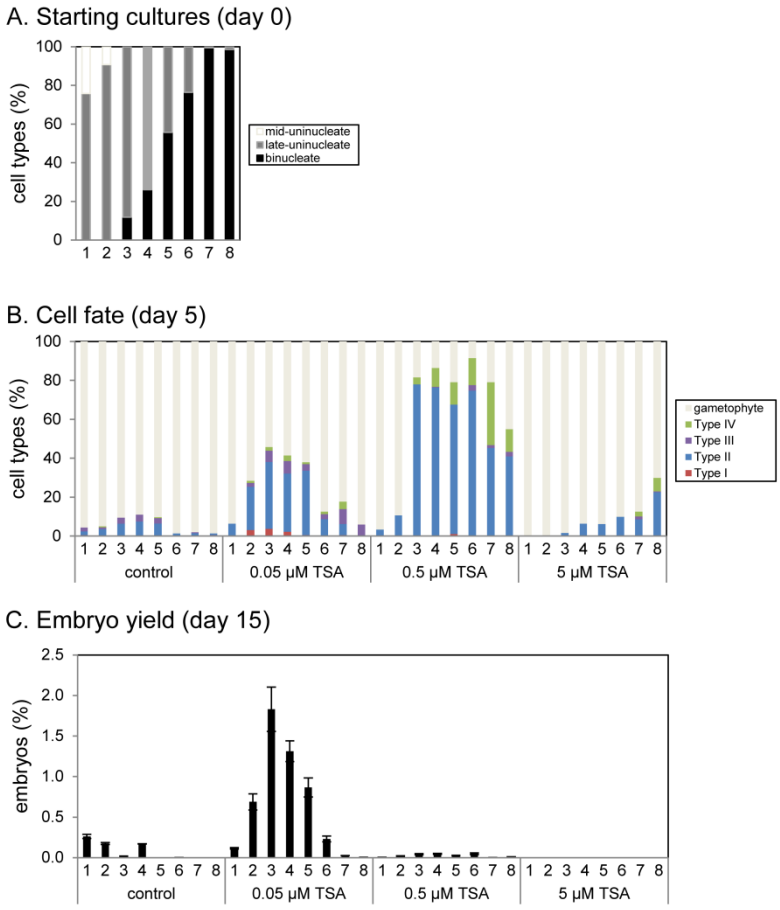
Supplemental data

The following materials are available at www.plantcell.org.

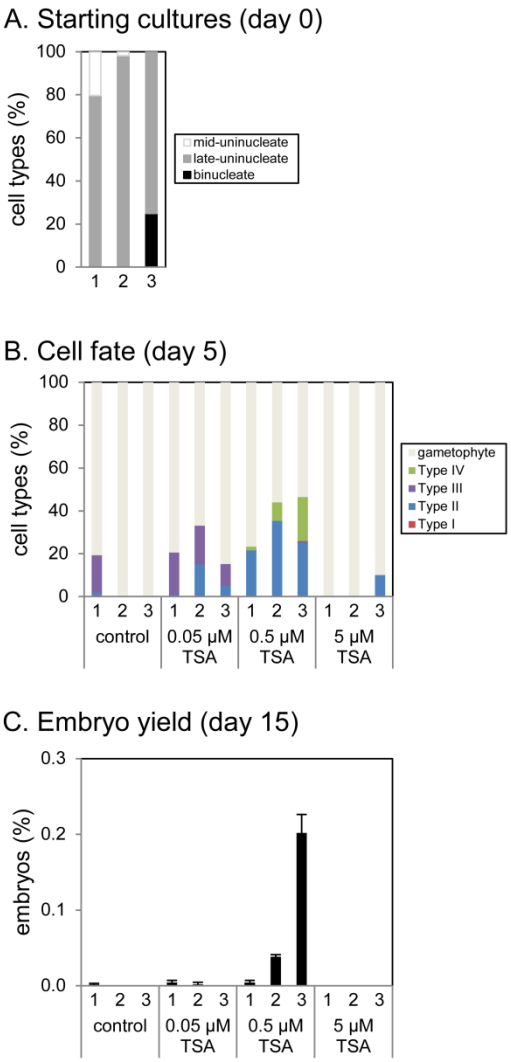


Supplemental Figure 1. Effect of the duration of TSA treatment on sporophytic cell division in *B. napus* microspore culture at 33 °C. **(A)** The developmental stage of microspores and pollen at the start of culture. For each treatment (1-2), the samples are ranked from left to right along the x-axis according to the developmental stage (youngest to oldest) of the microspores and pollen. **(B)** Sporophytic divisions in control and TSA-treated microspores/pollen from (**A**).

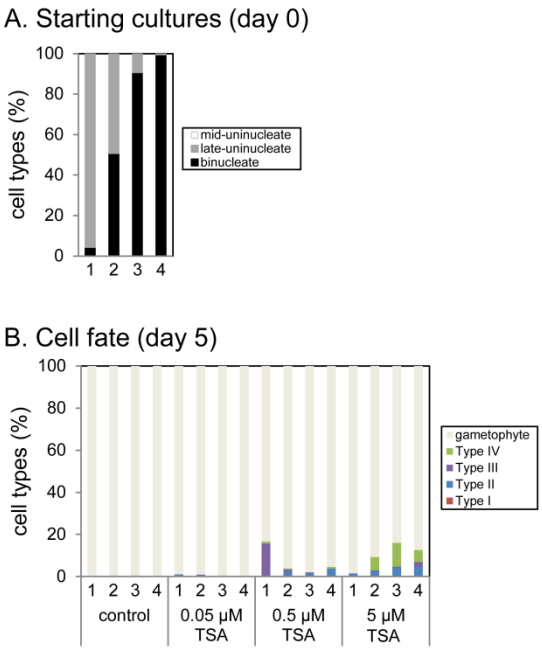


Supplemental Figure 2. Effect of TSA on cell fate and embryo formation in *B. napus* microspore culture at 33 °C.

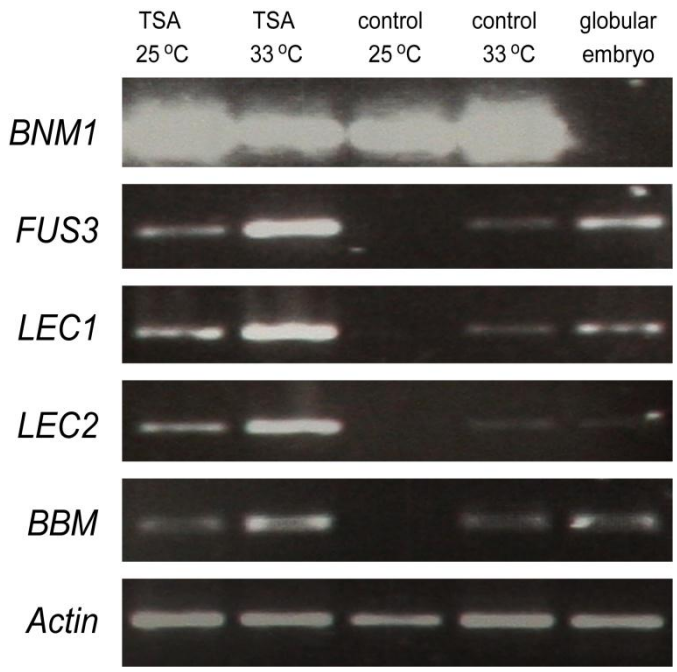
(A) The developmental stage of microspores and pollen at the start of culture. **(B)** The effect of TSA on cell fate in *B. napus* microspore embryo culture. Types I-IV are sporophytic. Dead microspores and pollen were not counted. **(C)** The effect of TSA on embryo yield. For each treatment (1-8), the samples are ranked from left to right along the x-axis according to the developmental stage (youngest to oldest) of the microspores and pollen.



Supplemental Figure 3. Effect of TSA on cell fate and embryo formation in *B. napus* microspore culture at 25 °C. **(A)** The developmental stage of microspores and pollen at the start of culture. **(B)** The effect of TSA on cell fate in *B. napus* microspore embryo culture. Types I-IV are sporophytic. Dead microspores and pollen were not counted. **(C)** The effect of TSA on embryo yield. For each treatment (1-3), the samples are ranked from left to right along the x-axis according to the developmental stage (youngest to oldest) of the microspores and pollen.

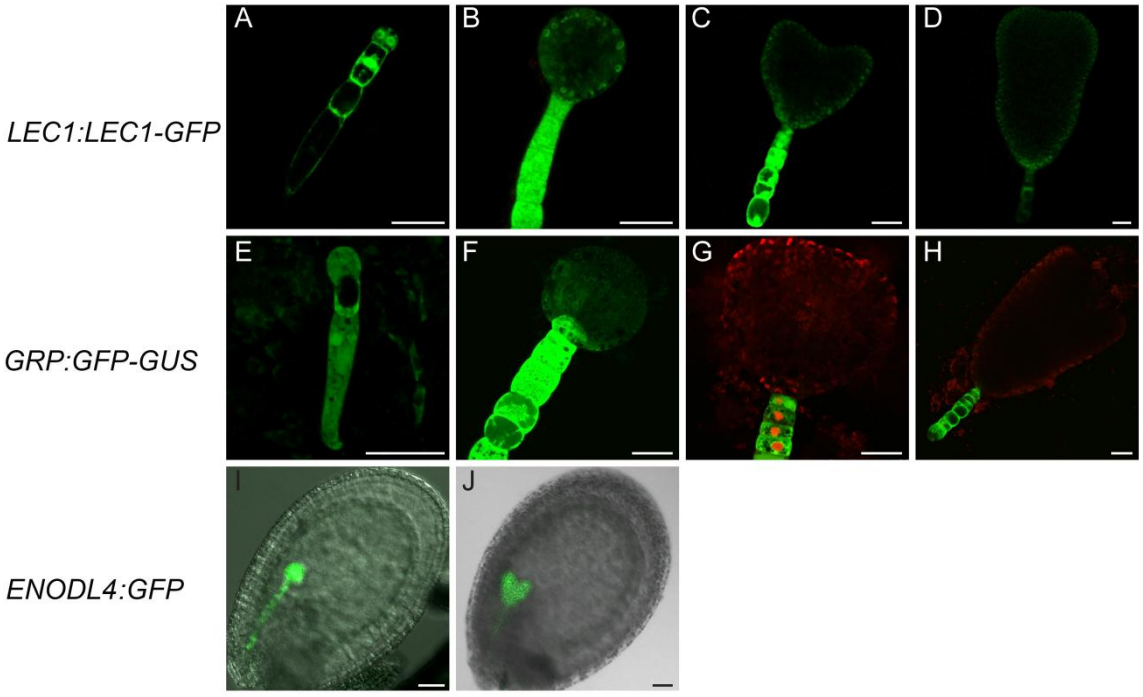


Supplemental Figure 4. Effect of TSA on cell fate and embryo formation in *B. napus* microspore culture at 18 °C. **(A)** The developmental stage of microspores and pollen at the start of culture. **(B)** The effect of TSA on cell fate in *B. napus* microspore embryo culture. Types I-IV are sporophytic. Dead microspores and pollen were not counted. Histodifferentiated embryos did not develop in control and TSA-treated samples that were cultured at 18 °C. For each treatment (1-4), the samples are ranked from left to right along the x-axis according to the developmental stage (youngest to oldest) of the microspores and pollen.



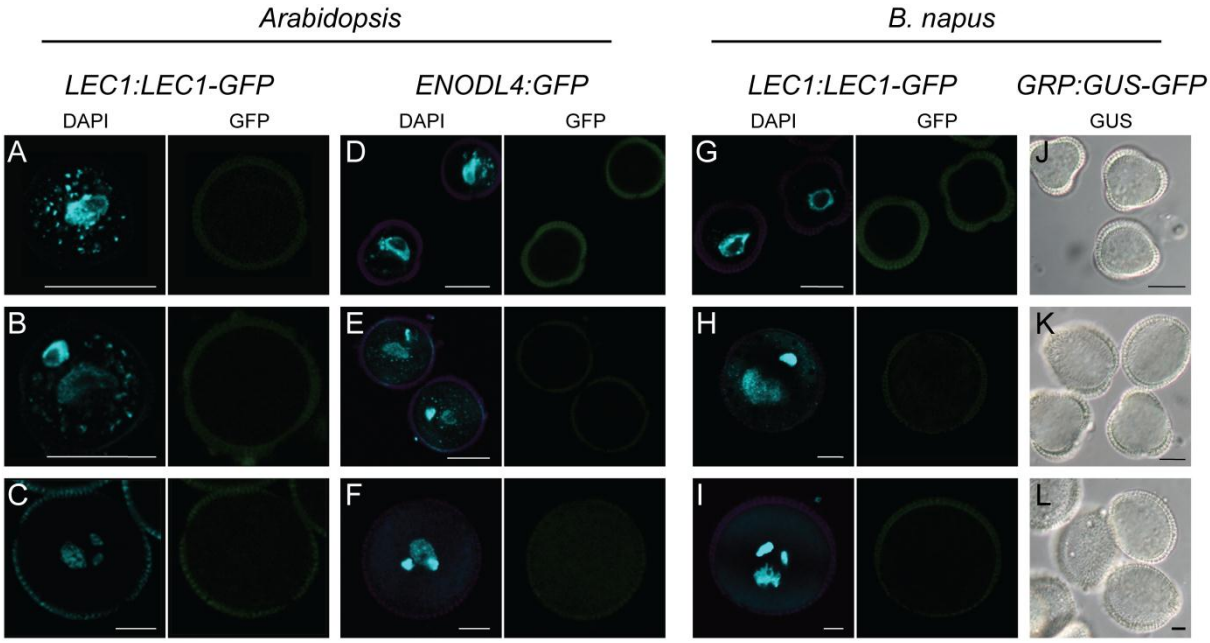
Supplemental Figure 5. Expression of cell fate markers in *B. napus* microspore culture.

RT-PCR analysis of the embryo-expressed genes, *FUSCA3* (*FUS3*), *LEAFY COTYLEDON1* (*LEC1*), *LEC2 and BABY BOOM* (*BBM*), and a pollen-expressed gene (*Brassica napus MICROSPORE1*; *BNM1*) in three day-old control and TSA-treated microspore cultures incubated at 33 °C and 25 °C. PCR amplification of actin was used to calibrate the amount of RNA per sample. Ten day-old globular stage microspore-derived embryos were used as a control. DNA primers are shown in Supplemental Table 5.



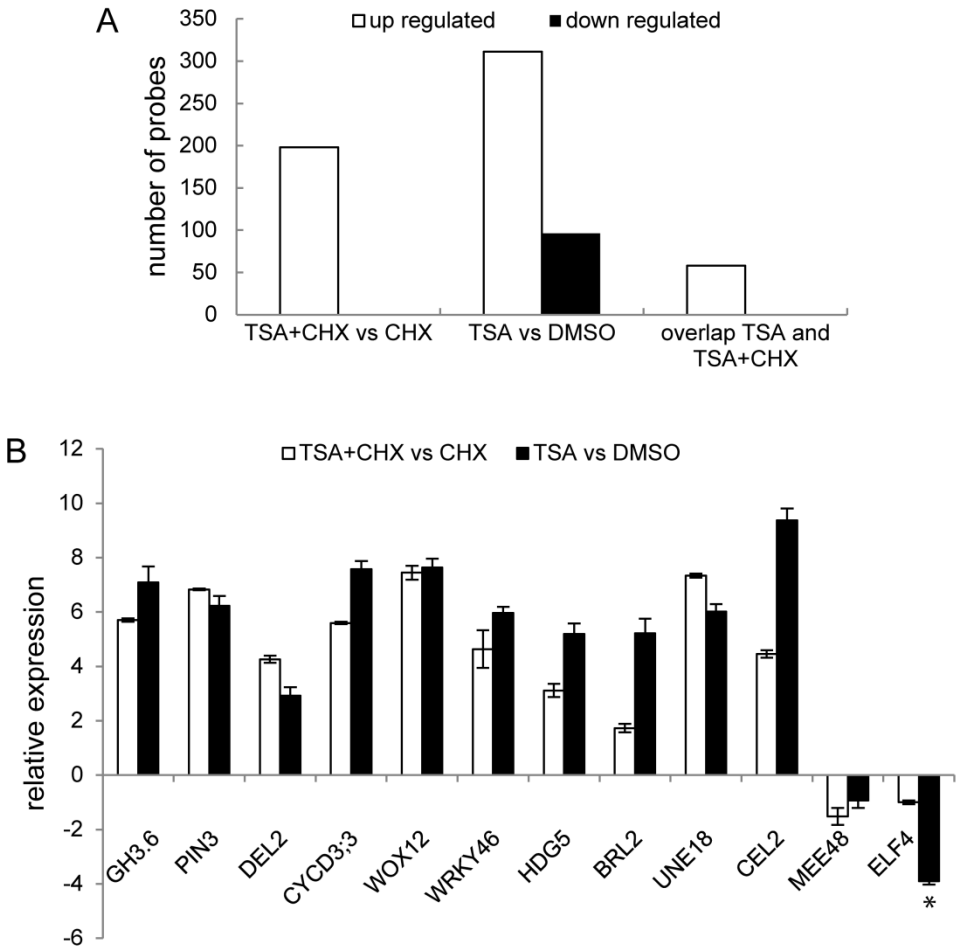
Supplemental Figure 6. Expression of the *LEC1, GRP* and *ENODL4* reporter lines in zygotic embryos.

(A-D) *LEC1:LEC1-GFP* expression in *B. napus*. **(A)** Two-celled embryo proper stage. **(B)** Globular embryo. At this stage, *LEC1* expression is focussed to the outer cell layers and the suspensor. **(C)** Heart stage embryo. **(D)** Torpedo stage embryo. **(E-H)** *GRP:GFP-GUS* expression in *B. napus*. **(E)** Zygote stage. **(F)** Late globular stage embryo. *GRP* expression gradually becomes restricted to the suspensor and its derivatives by the globular stage of development. **(G)** Transition stage embryo. **(H)** Torpedo stage embryo. **(I-J)** *ENODL4:GFP* expression in Arabidopsis. **(I)** Globular embryo. **(J)** Heart stage embryo. *ENODL4* expressed throughout the embryo proper and suspensor. Scale bar, 50 µm.



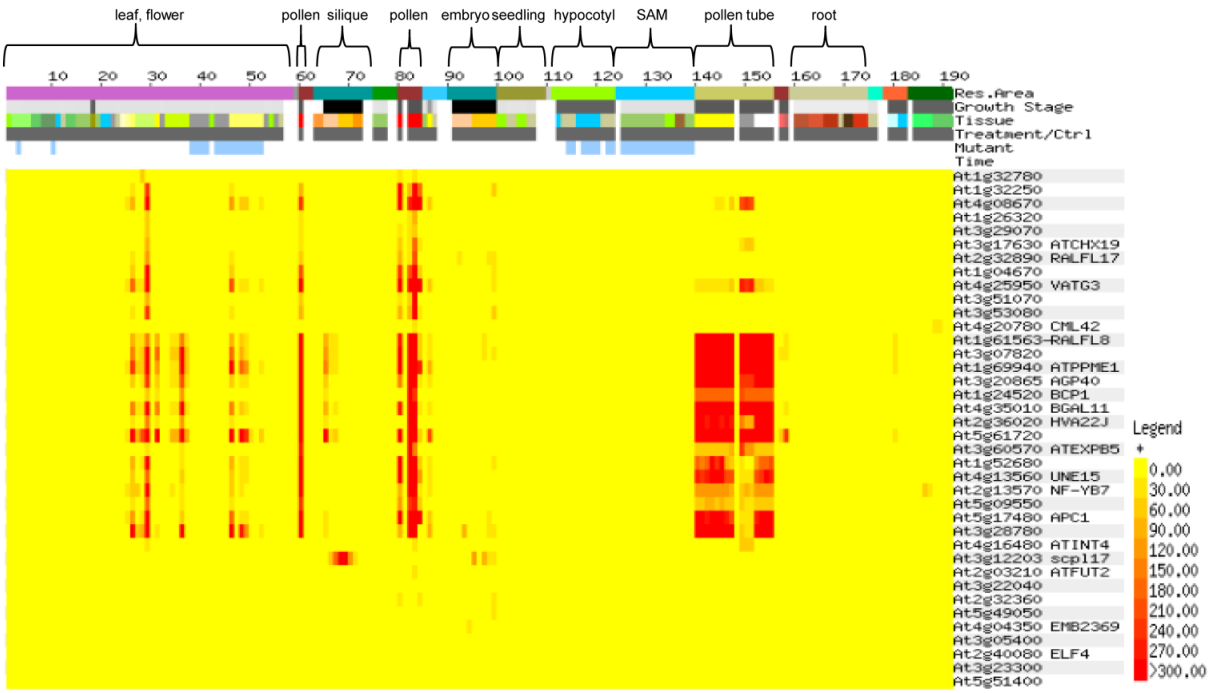
Supplemental Figure 7. Embryo reporters are not expressed during pollen development *in planta*.

(A-C) *LEC1:LEC1-GFP* expression in Arabidopsis. **(D-F)** *ENODL4:GFP* expression in Arabidopsis. **(G-I)** *LEC1:LEC1-GFP* expression in *B. napus*. **(J-L)** *GRP:GUS-GFP* expression in *B. napus*, visualized by GUS staining. **(A-I)** Nuclei are visualized by DAPI staining. Uninucleate (upper), binucleate (middle) and trinucleate (lower) stages of pollen development. Scale bar, 10 µm.



Supplemental Figure 8. Microarray analysis and validation.

(A) The number of probes that are significantly differentially-expressed (FDR<0.05) between TSA-treated or TSA and cycloheximide (CHX)-treated versus mock-treated *B. napus* microspore cultures, and that also showed at least a two-fold change in expression. **(B)** Validation of microarray gene expression data by quantitative real-time RT-PCR. *B. napus* microspore cultures were collected under the same conditions used for the microarray analysis. The relative expression of each gene after treatment with TSA or TSA plus CHX was calculated according to Livak and Schmittgen (2001) using the corresponding mock treatment as the calibrator and the *SAND* gene as the reference. *ELF4* expression (*) was down-regulated in the microarray analysis. The remaining genes were identified as upregulated genes in the microarray analysis. Relative expression is shown as $\log_2$ values ($-\Delta\Delta Ct$).



Supplemental Figure 9. TSA-down regulated genes are preferentially-expressed in pollen and pollen tubes. The data was compiled using the Bio-Analytic Resource for Plant Biology (BAR) web-based tool (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi) (Toufighi et al., 2005). A relatively high expression level is indicated by red and a relatively low expression level is by yellow.

References

Andrews, A.J., and Luger, K. (2011). Nucleosome structure(s) and stability: variations on a theme. Annu. Rev. Biophys. **40:** 99-117.

Bannister, A.J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res. **21:** 381-395.

Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992). New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol. Biol. **20:** 1195-1197.

Bennett, T., and Scheres, B. (2010). Root development - two meristems for the price of one? Curr. Top. Dev. Biol. **91:** 67-102.

Besnard, F., Vernoux, T., and Hamant, O. (2011). Organogenesis from stem cells *in planta*: multiple feedback loops integrating molecular and mechanical signals. Cell. Mol. Life Sci. **68:** 2885-2906.

Bicknell, R.A., and Koltunow, A.M. (2004). Understanding apomixis: recent advances and remaining conundrums. Plant Cell **16:** S228-S245.

Binarova, P., Hause, G., Cenklová, V., Cordewener, J.H., and Campagne, M.L. (1997). A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex. Plant Reprod. **10:** 200-208.

Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A.A., and Miki, B.L. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell **14:** 1737-1749.

Bouyer, D., Roudier, F., Heese, M., Andersen, E.D., Gey, D., Nowack, M.K., Goodrich, J., Renou, J.-P., Grini, P.E., and Colot, V. (2011). Polycomb repressive complex 2 controls the embryo-to-seedling phase transition. PLoS Genet. **7:** e1002014.

Bratzel, F., López-Torrejón, G., Koch, M., Del Pozo, J.C., and Calonje, M. (2010). Keeping cell identity in *Arabidopsis* requires PRC1 RING-Finger homologs that catalyze H2A monoubiquitination. Curr. Biol. **20:** 1853-1859.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature **391:** 597-601.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.-H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in *Arabidopsis*. Development **131:** 5263-5276.

Chen, Z., Hafidh, S., Poh, S.H., Twell, D., and Berger, F. (2009). Proliferation and cell fate establishment during *Arabidopsis* male gametogenesis depends on the Retinoblastoma protein. Proc. Natl. Acad. Sci. USA **106:** 7257-7262.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735-743.

Corral-Martínez, P., Parra-Vega, V., and 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. J. Exp. Bot. **64:** 3061-3075.

Custers, J.B.M. (2003). Microspore culture in rapeseed (*Brassica napus* L.). In Doubled Haploid Production in Crop Plants: A Manual, M. Maluszynski, K.J. Kasha, B.P. Forster, and I. Szarejko, eds (Dordrecht: Kluwer Academic Publishers), pp. 185-193.

Custers, J.B.M., Cordewener, J.H.G., Fiers, M.A., Maassen, B.T.H., van Lookeren Campagne, M.M., and Liu, C.M. (2001). Androgenesis in Brassica, a model system to study the initiation of plant embryogenesis. In Current Trends in the Embryology of Angiosperms. S.S. Bhojwani and W.Y. Soh, eds (Dordrecht: Kluwer Academic Publishers), pp. 451-470.

Daghma, D., Kumlehn, J., Hensel, G., Rutten, T., and Melzer, M. (2012). Time-lapse imaging of the initiation of pollen embryogenesis in barley (*Hordeum vulgare* L.). J. Exp. Bot. **63:** 6017-6021.

De Smet, I., and Beeckman, T. (2011). Asymmetric cell division in land plants and algae: the driving force for differentiation. Nat. Rev. Mol. Cell Biol. **12:** 177-188.

Dewitte, W., and Murray, J.A. (2003). The plant cell cycle. Annu. Rev. Plant Biol. **54:** 235-264.

Dewitte, W., Scofield, S., Alcasabas, A.A., Maughan, S.C., Menges, M., Braun, N., Collins, C., Nieuwland, J., Prinsen, E., and Sundaresan, V. (2007). Arabidopsis CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc. Natl. Acad. Sci. USA **104:** 14537-14542.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jürgens, G., and Estelle, M. (2005). Plant development is regulated by a family of auxin receptor F box proteins. Dev. Cell **9:** 109-119.

Ebel, C., Mariconti, L., and Gruissem, W. (2004). Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. Nature **429:** 776-780.

Fan, Z., Armstrong, K., and Keller, W. (1988). Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma **147:** 191-199.

Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R., and Pavletich, N.P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature **401:** 188-193.

Flaus, A., and Owen-Hughes, T. (2011). Mechanisms for ATP-dependent chromatin remodelling: the means to the end. FEBS J. **278:** 3579-3595.

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

Frank, M., Guivarc'h, A., Krupková, E., Lorenz-Meyer, I., Chriqui, D., and Schmülling, T. (2002). TUMOROUS SHOOT DEVELOPMENT (TSD) genes are required for co-ordinated plant shoot development. Plant J. **29:** 73-85.

Friml, J., Wisacute, J., Benková, E., Mendgen, K., and Palme, K. (2002). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature **415:** 806-809.

Gaj, M.D. (2004). Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul. **43:** 27-47.

González-Melendi, P., Testillano, P., Ahmadian, P., Fadón, B., and Risueño, M. (1996). New in situ approaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. Eur. J. Cell Biol. **69:** 373-386.

González, J., and Jouve, N. (2005). Microspore development during *in vitro* androgenesis in triticale. Biol. Plant. **49:** 23-28.

Gray-Mitsumune, M., and Matton, D.P. (2006). The *Egg apparatus 1* gene from maize is a member of a large gene family found in both monocots and dicots. Planta **223:** 618-625.

Gregoretti, I., Lee, Y.-M., and Goodson, H.V. (2004). Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J. Mol. Biol. **338:** 17-31.

Gresshoff, P.M., and Doy, C.H. (1972). Haploid *Arabidopsis thaliana* callus and plants from anther culture. Aust. J. Biol. Sci. **25:** 259-264.

Grozinger, C.M., and Schreiber, S.L. (2002). Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem. Biol. **9:** 3-16.

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

Gutierrez, C. (2009). The Arabidopsis cell division cycle. In The Arabidopsis Book, C. Somerville and E. Meyerowitz, eds (Rockville: American Society of Plant Biologists), pp. e0120.

Gutzat, R., Borghi, L., and Gruissem, W. (2012). Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. Trends Plant Sci. **17:** 139-148.

Halsall, J., Gupta, V., O'Neill, L.P., Turner, B.M., and Nightingale, K.P. (2012). Genes are often sheltered from the global histone hyperacetylation induced by HDAC inhibitors. PloS one **7:** e33453.

Harbour, J.W., and Dean, D.C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. **14:** 2393-2409.

Hause, B., Veenendaal, W.v., Hause, G., and Lammeren, A.v. (1994). Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv. Topas. Bot. Acta **107:** 407-415.

He, C., Chen, X., Huang, H., and Xu, L. (2012). Reprogramming of H3K27me3 is critical for acquisition of pluripotency from cultured *Arabidopsis* tissues. PLoS Genet. **8:** e1002911.

Henikoff, S., and Shilatifard, A. (2011). Histone modification: cause or cog? Trends Genet. **27:** 389-396.

Hollender, C., and Liu, Z. (2008). Histone deacetylase genes in *Arabidopsis* development. J. Integr. Plant Biol. **50:** 875-885.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. Scand. J. Stat. **6:** 65-70.

Ilić-Grubor, K., Attree, S.M., and Fowke, L.C. (1998). Comparative morphological study of zygotic and microspore-derived embryos of *Brassica napus* L. as revealed by scanning electron microscopy. Ann. Bot. **82:** 157-165.

Indrianto, A., Barinova, I., Touraev, A., and Heberle-Bors, E. (2001). Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. Planta **212:** 163-174.

Islam, S., and Tuteja, N. (2012). Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Sci. **182:** 134-144.

Iwai, H., Masaoka, N., Ishii, T., and Satoh, S. (2002). A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem. Proc. Natl. Acad. Sci. USA **99:** 16319-16324.

Jiang, C., and Pugh, B.F. (2009). Nucleosome positioning and gene regulation: advances through genomics. Nat. Rev. Genet. **10:** 161-172.

Johnston, A.J., Matveeva, E., Kirioukhova, O., Grossniklaus, U., and Gruissem, W. (2008). A dynamic reciprocal *RBR*-PRC2 regulatory circuit controls *Arabidopsis* gametophyte development. Curr. Biol. **18:** 1680-1686.

Joosen, R., Cordewener, J., Supena, E.D.J., Vorst, O., Lammers, M., Maliepaard, C., Zeilmaker, T., Miki, B., America, T., and Custers, J. (2007). Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol. **144:** 155-172.

Kaltchuk-Santos, E., Mariath, J.E., Mundstock, E., Hu, C.-y., and Bodanese-Zanettini, M.H. (1997). Cytological analysis of early microspore divisions and embryo formation in cultured soybean anthers. Plant Cell Tiss. Org. Cult. **49:** 107-115.

Krupková, E., and Schmülling, T. (2009). Developmental consequences of the *tumorous shoot development1* mutation, a novel allele of the cellulose-synthesizing KORRIGAN1 gene. Plant Mol. Biol. **71:** 641-655.

Krupková, E., Immerzeel, P., Pauly, M., and Schmülling, T. (2007). The *TUMOROUS SHOOT DEVELOPMENT2* gene of Arabidopsis encoding a putative methyltransferase is required for cell adhesion and co-ordinated plant development. Plant J. **50:** 735-750.

Lammens, T., Li, J., Leone, G., and De Veylder, L. (2009). Atypical E2Fs: new players in the E2F transcription factor family. Trends Cell Biol. **19:** 111-118.

Lichter, R. (1982). Induction of haploid plants from isolated pollen of *Brassica napus*. Z. Pflanzenphysiol. **105:** 427-434.

Loidl, P. (2004). A plant dialect of the histone language. Trends Plant Sci. **9:** 84-90.

Lotan, T., Ohto, M.-a., Yee, K.M., West, M.A., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1998). *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell **93:** 1195-1205.

Love, C.G., Graham, N.S., Lochlainn, S.Ó., Bowen, H.C., May, S.T., White, P.J., Broadley, M.R., Hammond, J.P., and King, G.J. (2010). A *Brassica* exon array for whole-transcript gene expression profiling. PloS one **5:** e12812.

Luo, M., Wang, Y.-Y., Liu, X., Yang, S., Lu, Q., Cui, Y., and Wu, K. (2012). HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*. J. Exp. Bot. **63:** 3297-3306.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature **391:** 601-605.

Makarevich, G., Leroy, O., Akinci, U., Schubert, D., Clarenz, O., Goodrich, J., Grossniklaus, U., and Köhler, C. (2006). Different Polycomb group complexes regulate common target genes in *Arabidopsis*. EMBO Rep. **7:** 947-952.

Malik, M.R., Wang, F., Dirpaul, J.M., Zhou, N., Polowick, P.L., Ferrie, A.M., and Krochko, J.E. (2007). Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol. **144:** 134-154.

Masubelele, N.H., Dewitte, W., Menges, M., Maughan, S., Collins, C., Huntley, R., Nieuwland, J., Scofield, S., and Murray, J.A. (2005). D-type cyclins activate division in the root apex to promote seed germination in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **102:** 15694-15699.

Miguel, C., and Marum, L. (2011). An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. J. Exp. Bot. **62:** 3713-3725.

Moehs, C.P., McElwain, E.F., and Spiker, S. (1988). Chromosomal proteins of *Arabidopsis thaliana*. Plant Mol. Biol. **11:** 507-515.

Moloney, M.M., Walker, J.M., and Sharma, K.K. (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. **8:** 238-242.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **96:** 13839-13844.

Preger, V., Tango, N., Marchand, C., Lemaire, S.D., Carbonera, D., Di Valentin, M., Costa, A., Pupillo, P., and Trost, P. (2009). Auxin-responsive genes *AIR12* code for a new family of plasma membrane b-type cytochromes specific to flowering plants. Plant Physiol. **150:** 606-620.

Pulido, A., Bakos, F., Castillo, A., Vallés, M., Barnabas, B., and Olmedilla, A. (2005). Cytological and ultrastructural changes induced in anther and isolated-microspore cultures in barley: Fe deposits in isolated-microspore cultures. J. Struct. Biol. **149:** 170-181.

Raghavan, V. (2004). Role of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2, 4-D. Am. J. Bot. **91:** 1743-1756.

Rampey, R.A., LeClere, S., Kowalczyk, M., Ljung, K., Sandberg, G., and Bartel, B. (2004). A family of auxin-conjugate hydrolases that contributes to free indole-3-acetic acid levels during Arabidopsis germination. Plant Physiol. **135:** 978-988.

Reynolds, T.L. (1993). A cytological analysis of microspores of *Triticum aestivum* (Poaceae) during normal ontogeny and induced embryogenic development. Am. J. Bot. **80:** 569-576.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J.A. (1999). Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science **283:** 1541-1544.

Riou-Khamlichi, C., Menges, M., Healy, J.S., and Murray, J.A. (2000). Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol. Cell. Biol. **20:** 4513-4521.

Rose, R., Mantiri, F., Kurdyukov, S., Chen, S., Wang, X., Nolan, K., and Sheahan, M. (2010). Developmental biology of somatic embryogenesis. In Plant Developmental Biology-Biotechnological Perspectives, P. E-C and D. MR, eds (Berlin Heidelberg: Springer), pp. 3-26.

Rossi, V., Locatelli, S., Lanzanova, C., Boniotti, M.B., Varotto, S., Pipal, A., Goralik-Schramel, M., Lusser, A., Gatz, C., and Gutierrez, C. (2003). A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription. Plant Mol. Biol. **51:** 401-413.

Sablowski, R. (2011). Plant stem cell niches: from signalling to execution. Curr. Opin. Plant Biol. **14:** 4-9.

Scholl, R., and Amos, J. (1980). Isolation of doubled-haploid plants through anther culture in *Arabidopsis thaliana*. Z. Pflanzenphysiol. **96:** 407-414.

Schubert, D., Clarenz, O., and Goodrich, J. (2005). Epigenetic control of plant development by Polycomb-group proteins. Curr. Opin. Plant Biol. **8:** 553-561.

Seguí-Simarro, J.M. (2010). Androgenesis revisited. Bot. Rev. **76:** 377-404.

Seguí-Simarro, J.M., and Nuez, F. (2008). How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol. Plant. **134:** 1-12.

Sozzani, R., Maggio, C., Giordo, R., Umana, E., Ascencio-Ibañez, J.T., Hanley-Bowdoin, L., Bergounioux, C., Cella, R., and Albani, D. (2010). The E2FD/DEL2 factor is a

component of a regulatory network controlling cell proliferation and development in Arabidopsis. Plant Mol. Biol. **72:** 381-395.

Staswick, P.E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M.T., Maldonado, M.C., and Suza, W. (2005). Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell **17:** 616-627.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2001). *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. USA **98:** 11806-11811.

Stone, S.L., Braybrook, S.A., Paula, S.L., Kwong, L.W., Meuser, J., Pelletier, J., Hsieh, T.-F., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2008). *Arabidopsis* LEAFY COTYLEDON2 induces maturation traits and auxin activity: implications for somatic embryogenesis. Proc. Natl. Acad. Sci. USA **105:** 3151-3156.

Sunderland, N. (1974). Anther culture as means of haploid induction. In Haploids in higher plants: advances and potential, K.J. Kasha, ed (Ontario: University of Guelph, Guelph), pp. 91–122.

Sunderland, N., and Wicks, F.M. (1971). Embryoid formation in pollen grains of *Nicotiana tabacum*. J. Exp. Bot. **22:** 213-226.

Tanaka, M., Kikuchi, A., and Kamada, H. (2008). The Arabidopsis histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. Plant Physiol. **146:** 149-161.

Tang, X., Liu, Y., He, Y., Ma, L., and Sun, M.-x. (2013). Exine dehiscing induces rape microspore polarity, which results in different daughter cell fate and fixes the apical–basal axis of the embryo. J. Exp. Bot. **64:** 215-228.

Tang, X., Lim, M.-H., Pelletier, J., Tang, M., Nguyen, V., Keller, W.A., Tsang, E.W., Wang, A., Rothstein, S.J., and Harada, J.J. (2012). Synergistic repression of the embryonic programme by SET DOMAIN GROUP 8 and EMBRYONIC FLOWER 2 in *Arabidopsis* seedlings. J. Exp. Bot. **63:** 1391-1404.

Telmer, C.A., Newcomb, W., and Simmonds, D.H. (1995). Cellular changes during heat shock induction and embryo development of cultured microspores ofBrassica napus cv. Topas. Protoplasma **185:** 106-112.

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L.A., Rhee, S.Y., and Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. **37:** 914-939.

Thomas, C., and Jiménez, V.M. (2006). Mode of action of plant hormones and plant growth regulators during induction of somatic embryogenesis: molecular aspects. In Somatic Embryogenesis, A. Mujib and J. Šamaj, eds (Berlin: Springer), pp. 157-175.

To, A., Valon, C., Savino, G., Guilleminot, J., Devic, M., Giraudat, J., and Parcy, F. (2006). A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. Plant Cell **18:** 1642-1651.

Touraev, A., Vicente, O., and Heberle-Bors, E. (1997). Initiation of microspore embryogenesis by stress. Trends Plant Sci. **2:** 297-302.

Wójcikowska, B., Jaskóła, K., Gąsiorek, P., Meus, M., Nowak, K., and Gaj, M.D. (2013). *LEAFY COTYLEDON2* (*LEC2*) promotes embryogenic induction in somatic tissues of Arabidopsis, via YUCCA-mediated auxin biosynthesis. Planta **238:** 425-440.

Xu, W., Parmigiani, R., and Marks, P. (2007). Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene **26:** 5541-5552.

Yang, X., and Zhang, X. (2010). Regulation of somatic embryogenesis in higher plants. Crit. Rev. Plant Sci. **29:** 36-57.

Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P., and Nielsen, E. (2006). High-affinity auxin transport by the AUX1 influx carrier protein. Curr. Biol. **16:** 1123-1127.

Zhang, C.L., McKinsey, T.A., Lu, J.-r., and Olson, E.N. (2001). Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. J. Biol. Chem. **276:** 35-39.

Zhang, H., and Ogas, J. (2009). An epigenetic perspective on developmental regulation of seed genes. Mol. Plant **2:** 610-627.

Zhang, H., Bishop, B., Ringenberg, W., Muir, W.M., and Ogas, J. (2012). The CHD3 remodeler PICKLE associates with genes enriched for trimethylation of histone H3 lysine 27. Plant Physiol. **159:** 418-432.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., and Dean, D.C. (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell **101:** 79-89.

Chapter 4

Histone Deacetylase Activity Regulates Microspore Embryo Induction and Differentiation

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Abstract

The male gametophyte of *Brassica napus* can be reprogrammed to form haploid embryos *in vitro* by exposing microspores and pollen to heat stress. Previously, we showed that treatment of microspore cultures with the histone deacetylase inhibitor (HDACi) trichostatin A (TSA), alone or in combination with heat stress, greatly enhances both the proportion of embryogenic microspores/pollen and the final embryo yield of these cultures. Here we extend this observation to a subset of HDACi that includes SAHA, scriptaid and oxamflatin and apicidin. In addition, we examined two phenomena that contribute to an efficient haploid embryo production system: 1) the differentiation of embryogenic cells into embryos and 2) the functionality or quality of these embryos. The majority of cell types in control and HDACi-treated cultures comprise embryogenic callus that fails to differentiate the tissues and organs normally found in *B. napus* embryos. We used microarray analysis to better understand why this embryogenic callus fails to differentiate. The transcriptomes of differentiating embryos and embryogenic callus were highly correlated, but showed differences in a small number of key genes; the expression of key regulators of embryo polarity and histodifferentiation was downregulated in the callus-like structures, as were genes involved in auxin and cytokinin signaling. Our data suggest that a subset of initially responsive microspores/pollen is not sufficiently committed by the heat-stress/HDACi treatment to complete the transition from initial embryogenic cell division to embryo morphogenesis. Finally, we observed that progressively older stages of donor microspores produced embryos of reduced quality, characterized by a progressive reduction in apical-basal patterning. Treatment with HDACi improved the quality of embryos produced from older gametophyte stages, by improving apical-basal patterning. The improved patterning was accompanied by stronger and broader basal expression of the *DR5* auxin response reporter. We propose that the auxin response of progressively older stages of microspores/pollen is repressed by an HDAC-dependent mechanism, and that this response is required to ensure proper embryo patterning. The initial histone acetylation status of microspores/pollen therefore plays a major role in both embryo induction and embryo morphogenesis in microspore culture.

Introduction

The DNA of plants, like most other eukaryotes, is wrapped around histones into nucleosomes, allowing it to be compacted into the nucleus. Cell cycle, developmental and environmental regulation of chemical modifications on histones provides a dynamic means to control the degree of DNA compaction and access of other proteins for replication and transcription. Among the various histone modifications, acetylation of lysine residues in the tails of the core histones has been extensively studied. Histone acetylation status is regulated by two groups of enzymes, histone acetyltransferases (HATs) and histone deacetylase (HDACs; Strahl and Allis, 2000). Hyper-acetylation of histones by HATs loosens chromatin structure by weakening the interaction between histones and DNA, and is associated with transcriptional activation, whereas hypo-acetylation of histones by HDACs compacts the chromatin structure and is associated with transcriptional repression (Millar and Grunstein, 2006).

Plant HDACs are classified into three families: the RPD3/HDA1 family, named after the yeast reduced potassium dependency 3 (Rpd3) and histone deacetylase 1 (hda1) proteins, the plant-specific HD2 or HD-tuin family, and the yeast sirtuin family, which is homologous to the yeast silent information regulator 2 (Sir2) proteins (Pandey et al., 2002; Hollender and Liu, 2008; Alinsug et al., 2009). The Rpd3/hda1 and HD2 families show zinc-dependent deacetylase activity (Finnin et al., 1999), while the sirtuin family shows nicotinamide adenine dinucleotide (NAD)-dependent deacetylase activity (Grozinger and Schreiber, 2002; Huang et al., 2007). Arabidopsis contains 18 HDAC genes (12 Rpd3/hda1, 4 HD2 and 2 SIRTUINS) and these have been shown to play major roles in plant development (Wu et al., 2000; Tian and Chen, 2001; Xu et al., 2005; Tanaka et al., 2008; Cigliano et al., 2013; Kim et al., 2013; Liu et al., 2013), as well gene silencing (Earley et al., 2006; Earley et al., 2010; Liu et al., 2012), and response to biotic/abiotic stress (Zhou et al., 2005; Kim et al., 2008; Wu et al., 2008; Luo et al., 2012a; Chen et al., 2013).

The activity of HDAC proteins can be controlled by chemical inhibitors, so-called HDAC inhibitors (HDACi). HDACi show selective sensitivity for the different HDAC families due to differences in their enzymatic mechanism. For example, HDACi that target Rpd3/hda1 and HD2-type HDACs inhibit HDAC activity by interacting with zinc ion and active site residues in the catalytic domain (Miller et al., 2003). This class of HDACi include short chain carboxylic acids, hydroxamic acids, benzamides, cyclic tetrapeptides and keto-derivatives (Bertrand, 2010). These different structural classes of HDACi also selectively inhibit different HDAC

proteins within a given class with varying efficiency (Blackwell et al., 2008). In plants, treatment with HDACi, mainly trichostatin A (TSA), has been used to determine the role of histone modification during seed germination (Tai et al., 2005; Tanaka et al., 2008), root development (Murphy et al., 2000; Xu et al., 2005; Nguyen et al., 2013), leaf development (Luo et al., 2012b), as well as in *in vitro* culture (Li et al., 2005; Furuta et al., 2011; Uddenberg et al., 2011).

Recently, we showed that histone acetylation plays a central role in the reprogramming of cultured male gametophytes to haploid embryo development (Li et al., 2014). During male gametophyte development, the haploid microspore divides to form a bicellular pollen grain that contains a vegetative nucleus and a smaller generative nucleus. The generative nucleus of the bicellular pollen grain divides once more, either in the anther or in the pollen tube during pollen germination, to form the trinucleate pollen grain comprising a vegetative cell and two smaller sperm cells (Twell, 2011). The microspores and bicellular pollen grains of many species can be induced to change developmental pathways *in vitro* from pollen development to embryo development after exposure to one or more physical or chemical stresses (Seguí-Simarro and Nuez, 2008; Soriano et al., 2013). This process is referred to as microspore embryogenesis, and is an example of the induced totipotency that characterizes many plant cells and tissues. In *Brassica napus*, microspore embryogenesis is induced by heat stress. We showed that treatment of cultured *B. napus* microspores/pollen with the HDACi TSA enhances embryogenic cell production and embryo yield compared to control cultures, and can even replace the requirement for heat-stress. Genetic analysis in Arabidopsis, which also exhibits TSA-mediated totipotency, showed that inhibition of HDA17 activity, together with additional uncharacterized HDACs may underlie the stressed-induced totipotency observed in cultured microspores and pollen (Li et al., 2014).

Microspore embryogenesis, besides being a model system for understanding plant cell totipotency, is widely used to accelerate the breeding process, as the haploid embryos produced in microspore culture can be converted to doubled-haploid (DH) plants, providing homozygous plants in a single generation (Forster et al., 2007; Ferrie and Möllers, 2011; Germanà, 2011). Microspore-derived embryo induction is an empirical process and the efficiency of embryo induction largely depends on the species and genotype. Currently, there are still many species and genotypes that remain recalcitrant for the different steps in DH production (Germanà, 2011). Many species and genotypes do not show any embryogenic

divisions in culture, while in other species, many of the embryogenic divisions lead to callus-like growth rather than embryo formation (Ferrie et al., 1995; Li et al., 2014). In addition to the low efficiency of embryo induction, the poor differentiation of embryos and the low rate of conversion of embryos into seedlings are also limiting factors for efficient DH production. A better understanding of the molecular and cellular basis of these processes, from embryo induction to embryo conversion, will not only contribute to our understanding cell totipotency at the fundamental level, but will also help to improve the efficiency of microspore embryogenesis systems at the practical level.

Here we show that, as with TSA treatment, treatment of *B. napus* microspore cultures with a range of HDACi that target Rpd3/hda1 and HD2 HDACs improved *B. napus* haploid embryo yield, not only by increasing the proportion of microspores and pollen that were reprogrammed to embryogenesis, but also by improving the quality of embryos obtained from suboptimal stages of donor pollen. The application of HDACi in microspore culture is therefore a powerful tool for understanding and improving haploid embryo production.

Results

A range of HDAC inhibitors enhance embryo induction and yield

Previously we showed that a short 20h TSA treatment enhances both embryogenic cell proliferation and embryo yield in *B. napus* microspore culture (Li et al., 2014). We determined whether other HDACi have a similar effect on microspore culture, by testing eight inhibitors with specificity for RPD3/HDA1 and HD-tuin/HD2-type HDACs, namely seven hydroxymates and one cyclic tetrapeptide (Supplemental Table I). We examined the effect of these HDACi on two parameters, embryogenic cell divisions and final embryo yield, using the *B. napus* doubled-haploid line DH12075, which shows a poor response in microspore culture. The heat-stressed cultures are hereafter referred to as the control cultures and the heat stress plus HDACi-treated culture as HDACi cultures.

Four types of embryogenic (sporophytic) multicellular structures are normally observed in five-day old control and HDACi-treated cultures (Figure 1A, Li et al., 2014): Type I structures, which are compact and still enclosed in the pollen exine; Type II compact callus-like structures, in which the exine shows partially early rupture; Type III structures, in which the sporophytically divided cells have been extruded from the exine and; Type IV loose callus-

like structures, in which the exine has completely ruptured (Figure 1A). The cells from Type II and IV structures are more loosely connected than those of Type I structures, with Type IV cells being more loose than Type II cells. All of these cell types express embryo markers (Li et al., 2014), and although Types I, II and IV continue to divide in culture, only Type I structures are thought to form histodifferentiated embryos. These four cell types were used as measure for the effect of HDACi treatment on initial embryogenic growth.

Most of the HDACi, with the exception of SAHA, SBHA, APHA8 and tubacin, had a positive, concentration- and stage-dependent effect on either the proportion or quality (Type I vs callus) of embryogenic divisions, as compared to the control (Figure 1B-G; Supplemental Data Set 1). Of the HDACi with a positive effect, all, with the exception of oxamflatin, were most effective in inducing embryogenic cell divisions from initial gametophyte cell populations that contained a mixture of late uninucleate microspores (85%) and early binucleate pollen (15%). Most of the HDACi with a positive effect on embryogenic cell divisions mainly induced Type II compact callus-like structures. Notably, treatment with 5 μM oxamflatin induced a relatively high proportion of the Type I embryogenic divisions (up to 14%; Figure 1G) that are correlated with histodifferentiated embryo formation.

These data indicate that a wide range of HDACi improved embryogenic cell division in *B. napus* microspore culture. The inability of certain HDACi to improve embryo induction suggests that they are either active at different concentrations than were tested here, or that they do not target the specific HDACs that repress microspore/pollen totipotency.

Embryogenic callus is characterized by misexpression of hormone and patterning genes

Treatment of microspore cultures with TSA and other HDACi greatly enhanced embryogenic cell proliferation compared to control cultures. As in control cultures, the vast majority of embryogenic structures in HDACi-treated cultures are callus-like. These callus-like structures express the *LEAFY COTYLEDON 1* (*LEC1*) and *GLYCINE-RICH PROTEIN* (*GRP*) embryo reporters (Li et al., 2014; Soriano et al., 2014/Chapter 5), indicating that they have entered the embryo development pathway, but upon further culture remain as loosely connected cell clusters that do not seem to develop into histodifferentiated embryos i.e. embryos with clear root and shoot poles and cotyledons.

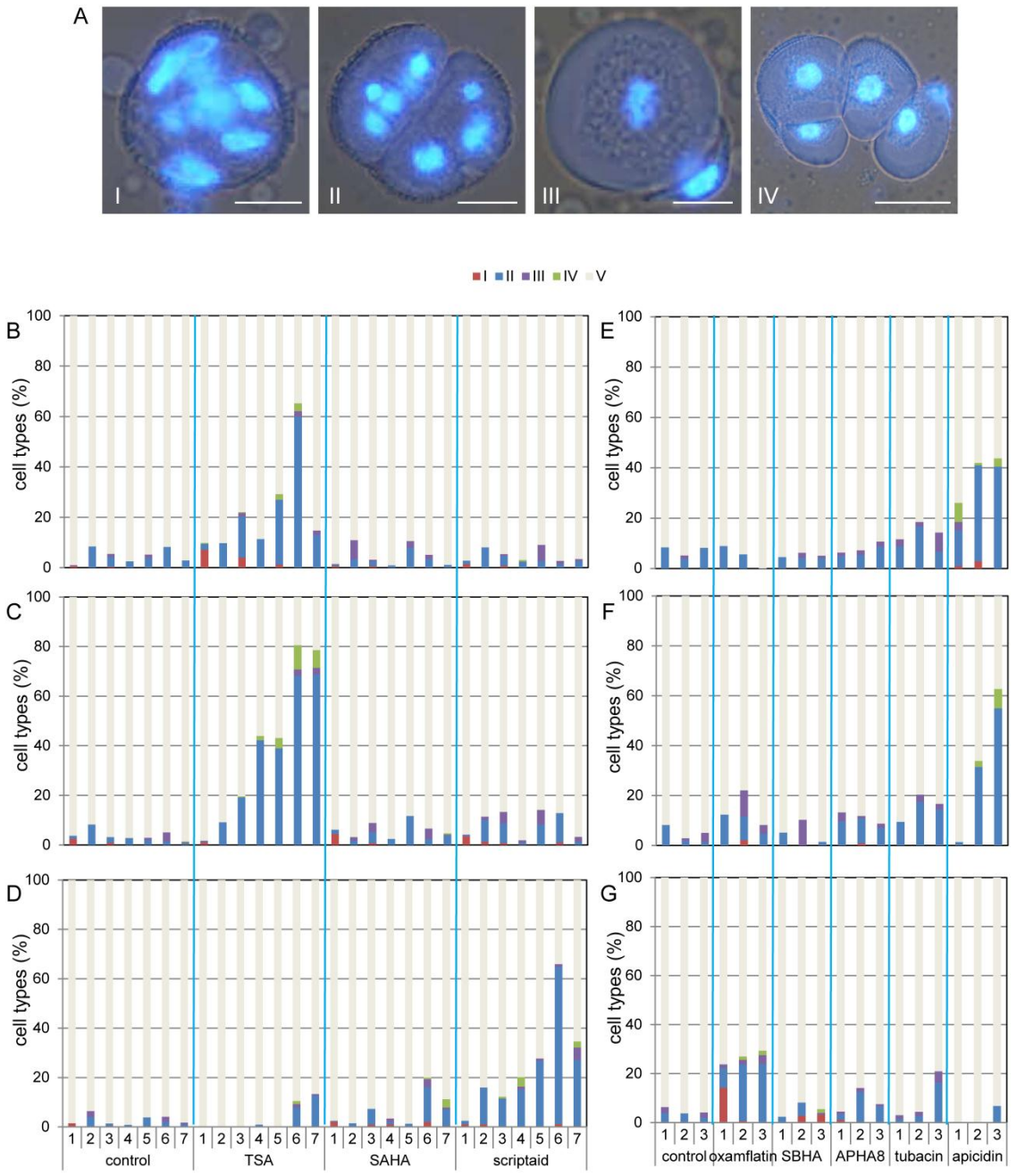

Figure 1. HDAC inhibitors enhance embryogenic cell divisions in *B. napus* microspore culture.

(A) Embryogenic structures found in control and HDAC inhibitor-treated DH12075 cultures. The sporophytic cell clusters are categorized as follows: Type I, classical embryo-forming structures (red bars); Type II, compact callus-like structures (blue bars); Type III, extruded sporophytic structures (purple bars) and Type IV, loose callus-like structures (green bars). Dead microspores and pollen were not included. Control, DMSO-treated sample. **(B-G)** The percentage of cells that had divided gametophytically (grey bars) or sporophytically (coloured bars) after five days of microspore culture in control and HDAC inhibitor-treated cultures. The cell types corresponding to the coloured bars (I-IV) are shown in **(A)**. The developmental stages of the starting material are ranked from youngest to oldest (1-7 or 1-3), as described in Supplemental Data Set 1. The rankings are not absolute, but rather relative within one data set. Three concentrations of HDAC inhibitors were evaluated: 0.05 µM (**B** and **E**), 0.5 µM (**C** and **F**) and 5 µM (**D** and **G**). Scale bars, 50µm.

We examined the gene expression profiles of globular stage embryos and embryogenic callus to obtain more insight into the molecular pathways underlying the development of differentiated embryos and embryogenic callus. Enriched samples containing a high proportion of seven day-old Type I globular-stage embryos (at least 93%; Supplemental Figure 1A; Supplemental Data Set 1) were obtained by inducing microspore cultures of the highly embryogenic *B. napus* genotype Topas DH4079 with heat-stress. Enriched samples of Type II and IV embryogenic callus (at least 97%) were obtained by inducing microspore cultures of the same genotype with heat-stress in combination with a 20h treatment with 0.5 µM TSA (Supplemental Figure 1A; Supplemental Data Set 1). At seven days of culture, control globular-stage embryos have burst out of the surrounding exine and have just started to show morphological and cellular-molecular signs of histodifferentiation (Soriano et al., 2014/Chapter 5). In contrast, callus-like structures had either completely burst out of the exine or showed broken patches of exine, and do not show any morphological signs of patterning.

Scatterplot analysis of the gene expression values from globular embryos and embryogenic callus showed that the samples were highly similar (R^2 =0.938, p-value <$2.2*10^{-6}$) (Supplemental Figure 1B). Microarray analysis of these samples identified 1168 probe sets (752 genes) that were significantly differentially-expressed more than two fold ($\log_2$ ratio ≤-1 or ≥1, FDR≤0.05). All the genes showing significant differential expression were characterized by relatively low expression (close to detection threshold) in one tissue (Supplemental Figure 1B, Supplemental Data Set 2). Of these, 703 probe sets (441 genes) were down-regulated in embryogenic callus compared to globular stage embryos (Supplemental Data Set 2). Gene Ontology (GO) analysis using BiNGO (Maere et al., 2005) showed that probe-sets corresponding to genes with functions related to development (embryo, meristem, patterning) and response to stimuli (abiotic, organic substances, hormones) and signalling (phosphate/phosphorylation) were significantly overrepresented among the down-regulated genes (Supplemental Figure 1D, Supplemental Data Set 2). 465 probe sets (311 genes) were up-regulated in embryogenic callus compared to globular stage embryos, and these were enriched in the GO categories peptide transport and secondary metabolism (amino acid, lignin, phenylpropanoid and cellulose) (Supplemental Figure 1E, Supplemental Data Set 2). The differential expression of a selection of genes was confirmed using quantitative real-time RT-PCR (Supplemental Figure 1C).

Closer inspection of the genes in each of the functional categories showed that genes with roles in embryo patterning and/or meristem development were down regulated in embryogenic callus compared to globular stage embryos (Table I). These include the *WUSCHEL RELATED HOMEOBOX* (*WOX*) *genes, WOX*2, *PRESSED FLOWER* (*PRS*)/*WOX3*, *WOX5*, and *STIMPY* (*STIP*)/*WOX9* (Matsumoto and Okada, 2001; Haecker et al., 2004; Sarkar et al., 2007), *TARGET OF MONOPTEROS7* (*TMO7*, Schlereth et al., 2010), *HANABA TARANU* (*HAN*)*/MONOPOLE* (*MNP*, Nawy et al., 2010), *EMBRYO MAKER (EMK)/CHOTTO* (*CHO*)/*AINTEGMENTA-like 5* (*AIL5*, Tsuwamoto et al., 2010), *PLETHORA2 (PLT2,* Galinha et al., 2007), *BABY BOOM* (*BBM*; Boutilier et al., 2002), *JACKDAW* (*JKD*, Hassan et al., 2010). Some of the down-regulated genes are also involved in apical pole specification, such as *DORNRÖSCHEN* (*DRN)* and *DRNL* (Chandler et al., 2007; Chandler et al., 2011), *PHABULOSA* (*PHB*, McConnell et al., 2001), *ALTERED MERISTEM PROGRAM1* (*AMP1*, Nogué et al., 2000) and *CLAVATA3* (Clark et al., 1995). Some of the down-regulated genes are also involved in epidermal specification. For example, the *HOMEODOMAIN GLABROUS (HDG)* genes, *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*; Abe et al., 2003) and *HDG11* (Nakamura et al., 2006), as well as *PROTODERMAL FACTOR 1* (*PDF1*, Abe et al., 1999), *ARABIDOPSIS CRINKLY 4* (*ACR4*, Tanaka et al., 2002), *GASSHO 1* (*GSO1*) and *GSO2* (Tsuwamoto et al., 2008), and *SCRAMBLED* (*SCM*, Kwak and Schiefelbein, 2007). However, we also observed that expression of two members of this family, *HDG2* and *HDG3*, was up-regulated.

In addition to down-regulation of genes involved in embryo patterning and meristem development, we also observed down-regulation of genes involved in auxin signalling and transport, including the auxin response factors (*ARF*) *ARF5*/*MONOPTEROS* (*MP*)*, ARF8, ARF18* (Berleth and Jurgens, 1993; Rademacher et al., 2011), repressors of ARF gene expression, the Aux/IAA transcriptional repressors *INDOLE-3-ACETIC-ACID2* (*IAA2*)*, IAA19, IAA29, IAA30* (Overvoorde et al., 2005), and the auxin efflux carrier genes *PIN-FORMED3* (*PIN3*), *PIN4* and *PIN7* (Friml et al., 2003).

Genes involved in cytokinin signal transduction were also down regulated. For example, *ARABIDOPSIS RESPONSE REGULATOR7* (*ARR7*) and *ARR15* (Müller and Sheen, 2008), which belong to type-A ARRs and are negative regulators of cytokinin signalling, and *CYTOKININ RESPONSE FACTOR 1* (*CRF1*) and *CRF3* (Rashotte et al., 2006; Xu et al., 2012), which are positive regulators of cytokinin response. Overexpression of *CRF3* also induces callus

formation (Xu et al., 2012). One cytokinin-regulated cell cycle gene *CYCLIN D3;1* (*CYCD3;1*) was also down regulated (Riou-Khamlichi et al., 1999).

Among the genes that were up-regulated in embryogenic callus-like structures, were genes involved in cytokinin signalling, including two type-B cytokinin response regulators *ARR19* and *ARR21*, which act as positive regulators of cytokinin signalling, and a type-C regulator *ARR22,* which acts as a negative regulator of cytokinin signalling. Overexpression of a dominant-negative form of *ARR21* induces callus formation in Arabidopsis (Tajima et al., 2004). Expression of *CYTOKININ OXIDASE 5* (*CKX5*), which encodes a cytokinin oxidase that mediates cytokinin degradation (Bartrina et al., 2011), was also upregulated.

Embryo patterning, root/shoot meristem and auxin/cytokinin transport and signalling are intertwined processes that control the major cell fate and histodifferentiation events in zygotic embryos. Our results suggest that embryogenic callus formation is associated with a global misregulation of these processes.

Table I. Significantly differentially-expressed genes with known functions

Down-regulated genes		
Locus	AGI	References
Auxin-related		
AUXIN RESPONSE FACTOR5 (ARF5)/MONOPTEROS (MP)	At1g19850	Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Ulmasov et al., 1999; Weijers et al., 2006
AUXIN RESPONSE FACTOR8 (ARF8)	At5g37020	Nagpal et al., 2005
AUXIN RESPONSE FACTOR18 (ARF18)	At3g61830	Rademacher et al., 2011
INDOLE-3-ACETIC-ACID2 (IAA2)	At3g23030	Overvoorde et al., 2005
INDOLE-3-ACETIC-ACID19 (IAA19)	At3g15540	
INDOLE-3-ACETIC-ACID29 (IAA29)	At4g32280	
INDOLE-3-ACETIC-ACID30 (IAA30)	At3g62100	
PIN-FORMED3 (PIN3)	At1g70940	Friml et al., 2003; Benková et al., 2003
PIN-FORMED4 (PIN4)	At2g01420	
PIN-FORMED7 (PIN7)	At1g23080	
Development		
WUSCHEL RELATED HOMEOBOX2 (*WOX*2)	At5g59340	Haecker et al., 2004; Breuninger et al., 2008
*PRESSED FLOWER (PRS)/WUSCHEL RELATED HOMEOBOX*3 (*WOX3*)	At2g28610	Matsumoto and Okada, 2001
*WUSCHEL RELATED HOMEOBOX*5 (*WOX5*)	At3g11260	Sarkar et al., 2007
*WUSCHEL RELATED HOMEOBOX*9 (*WOX9*)/STIMPY (*STIP*)	At2g33880	Wu et al., 2007
TARGET OF MONOPTEROS7 (TMO7)	At1g74500	Schlereth et al., 2010
HANABA TARANU (HAN)/MONOPOLE (MNP)	At3g50870	Zhao et al., 2004; Nawy et al., 2010

PLETHORA2 (PLT2)	At1g51190	Aida et al., 2004; Boutilier et al., 2002; Galinha et al., 2007
BABY BOOM (BBM)/PLT4	At5g17430	
PLT5/AINTEGMENTA-like5 (AIL5)/EMBRYOMAKER (EMK)	At5g57390	Tsuwamoto et al., 2010
ALTERED MERISTEM PROGRAM1 (AMP1)	At3g54720	Chaudhury et al., 1993; Nogue et al., 2000a; Nogue et al., 2000b
CLAVATA3/ESR-RELATED17 (CLE17)	At1g70895	Wang and Fiers, 2010; Clark et al., 1995
DORNRÖSCHEN (*DRN)/ENHANCER OF SHOOT REGENERATION1 (ESR1)*	At1g12980	Cole et al., 2009; Chandler et al., 2007, 2011
DORNRÖSCHEN-LIKE (DRNL)/ ENHANCER OF SHOOT REGENERATION2 (ESR2)	At1g24590	
PHABULOSA (PHB)	At2g34710	McConnell et al., 2001
JACKDAW (JKD)	At5g03150	Hassan et al., 2010
Epidermis development		
Arabidopsis thaliana MERISTEM LAYER1 (ATML1)	At4g21750	Lu et al., 1996; Sessions et al., 1999; Abe et al., 2001; Abe et al., 2003; Takada et al., 2013
PROTODERMAL FACTOR1 (PDF1)	At2g42840	Abe et al., 1999
ARABIDOPSIS CRINKLY4 (ACR4)	At3g59420	Tanaka et al., 2002
GASSHO1 (GSO1)	At4g20140	Tsuwamoto et al., 2008
GASSHO2 (GSO2)	At5g44700	
HOMEODOMAIN GLABROUS11(HDG11)	At1g73360	Nakamura et al., 2006
SCRAMBLED (SCM)	At1g11130	Kwak and Schiefelbein, 2007
Cell cycle		
CYCLIN D3;1 (CYCD3;1)	At4g34160	Riou-Khamlichi et al., 1999
Cytokinin-related		
ARABIDOPSIS RESPONSE REGULATOR7 (ARR7)	At1g19050	Lee et al., 2007; Müller and Sheen, 2008
ARABIDOPSIS RESPONSE REGULATOR15 (ARR15)	At1g74890	Kiba et al., 2003; Müller and Sheen, 2008
CYTOKININ RESPONSE FACTOR 1 (CRF1)	At4g11140	Rashotte et al., 2006; Xu et al., 2012
CYTOKININ RESPONSE FACTOR 3 (CRF3)	At5g53290	
Cytoskeleton		
MICROTUBULE-ASSOCIATED PROTEINS65-1 (MAP65-1)	At5g55230	Lucas et al., 2011; Lucas and Shaw, 2012
MICROTUBULE-ASSOCIATED PROTEINS65-2 (MAP65-2)	At4g26760	

Up-regulated genes		
Auxin-related		
GH3-10/DWARF IN LIGHT 2 (DFL2)	At4g03400	Ulmasov et al., 1995
PIN2/ETHYLENE INSENSITIVE ROOT 1 (EIR1)	At5g57090	Friml et al., 2003; Benková et al., 2003
Development		
WUSCHEL RELATED HOMEOBOX 14 (WOX14)	At1g20700	Etchells et al.,2013
LATERAL ORGAN BOUNDARY DOMAIN18 (LBD18)	At2g45420	Lee et al., 2009; Berckmans et al., 2011
GROWTH-REGULATING FACTOR 4 (GRF4)	At3g52910	Kim et al., 2003
AGAMOUS-LIKE 80 (AGL80)	At5g48670	Portereiko et al., 2006
NGATHA4 (NGA4)	At4g01500	Trigueros et al., 2009
TRANSPARENT TESTA16 (TT16)/ABS/AGL32	At5g23260	Nesi et al., 2002

MADS AFFECTING FLOWERING 4 (MAF4)	At5g65070	Ratcliffe et al., 2003
MYB DOMAIN PROTEIN 56 (MYB56)	At5g17800	Zhang et al., 2013
MYB DOMAIN PROTEIN 63 (MYB63)	At1g79180	Zhou et al., 2009
Epidermis development		
HOMEODOMAIN GLABROUS 3 (HDG3)	At2g32370	Nakamura et al., 2006
HOMEODOMAIN GLABROUS 2 (HDG2)	At1g05230	Peterson et al., 2013
ENHANCER OF GLABRA 3 (EGL3)	At1g63650	Bernhardt et al., 2003, 2005; Zhang et al., 2003
Cytokinin-related		
CYTOKININ OXIDASE 5 (CKX5)	At1g75450	Bartrina et al., 2011
ARABIDOPSIS RESPONSE REGULATOR 22 (ARR22)	At3g04280	Horák et al., 2008
ARABIDOPSIS RESPONSE REGULATOR 21 (ARR21)	At5g07210	Tajima et al., 2004
ARABIDOPSIS RESPONSE REGULATOR 19 (ARR19)	At1g49190	Day et al., 2008

Inhibition of HDAC activity improves embryo yield and quality

Previously, we showed that TSA treatment increases the range of pollen developmental stages that form differentiated embryos in microspore culture (Li et al., 2014). We therefore examined additional HDACi for their ability to enhance embryo production in *B. napus* DH12075. As with TSA, all HDACi significantly enhanced embryo production compared to control cultures, although embryo formation was less efficient after treatment with certain HDACi (SBHA, APHA8, tubacin, apicidin) than with TSA (Figure 2). The effect of each HDACi on embryo yield was stage and concentration-dependent (Figure 2). For example, as shown previously, treatment with high concentrations of TSA had a negative effect on embryo yield (Figure 2C, Li et al., 2014). Enhanced embryo formation was observed over a wider range of developmental stages, thereby contributing to an even larger improvement in embryo yield. We focussed our further experiments on the HDACi TSA, SAHA and scriptaid.

Treatment of microspore cultures with HDACi also improved the quality of the embryos that were formed. Four major types of embryos were observed in control cultures (Figures 2 and 3; Supplemental Table II; supplemental Data Set 1). These comprised bipolar embryos with two cotyledons, a long embryonic axis and a clear root pole (referred to as normal embryos) (Figure 3A), along with less well-formed embryos, including embryos with a rough hypocotyl surface and reduced cotyledons (Figure 3B; referred to as rough embryos), embryos with reduced apical and basal poles (Figure 3C; referred to as reduced embryos), and ball-shaped embryos with or without small round cotyledons (Figure 3D). These ball shaped embryos differ from embryogenic callus in that they are compact structures, rather than clusters of loosely connected cells. Embryo morphology was largely dependent on the

developmental stage of the microspores/pollen used to initiate the culture, with older stages generating progressively poorer quality embryos (Supplemental Table II). Only one type of embryo was found within each developmental range of microspore/pollen development. A similar trend of poorer embryo morphology in cultures derived from older gametophytes was observed in HDACi-treated cultures, except that (concentration-dependent) treatment with HDACi improved embryo morphology at each stage compared to the control (Figure 3E-H; Supplemental Table II). The major morphological improvements obtained after the HDACi treatments were smoothening of the epidermis, elongation of the embryonic axis and growth of the apical pole (Figure 3F-H). After HDACi treatment the rough embryos in control cultures were replaced by elongated and less rough embryos (referred to as wrinkled; Figure 3B vs F), while the reduced embryos in control cultures were replaced by elongated embryos with fused cotyledons (Figure 3C vs G), and the ball-shaped clusters were replaced by reduced embryos (Figure 3D vs H). At higher concentrations, two inhibitors, scriptaid and TSA, induced the formation of embryos with fused cotyledons and/or pin-shaped embryos with severely reduced cotyledons.

To determine whether embryos from HDACi-treated cultures could be converted into seedlings, we placed embryos from TSA, SAHA and scriptaid-treated cultures on solid germination medium and observed the timing and efficiency of root and shoot formation compared to control cultures (Supplemental Data Set 1; Figure 4). All embryos except ball-shaped embryos, developed a single root from the basal tip of the embryo within five days of plating (Figure 4A-G), suggesting that these roots were derived directly from a meristem, rather than indirectly through the slower process of callus formation and organogenesis. Notably, the root of embryos from HDACi-treated cultures (Figure 4D-G) grew faster compared to embryos from control cultures (Figure 4A-C). Callus formed at the junction of the hypocotyl and root of all embryos, especially pin-shaped embryos from TSA treated cultures, which also formed secondary embryos from the enlarged hypocotyl (Figure 4G). Shoot formation was evaluated 20 days after germination in embryos from control cultures and HDACi-treated cultures (Figure 4H; Supplemental Data Set 1). Insufficient embryos were available in the 0.05 μM scriptaid and SAHA treatments to examine the effect of these HDACi on shoot development. Around 20-30% of the embryos from control and HDACi-treated cultures formed shoots, with the exception of ball-shaped embryos (control and HDACi cultures), pin-shaped embryos (TSA cultures) and reduced embryos from most

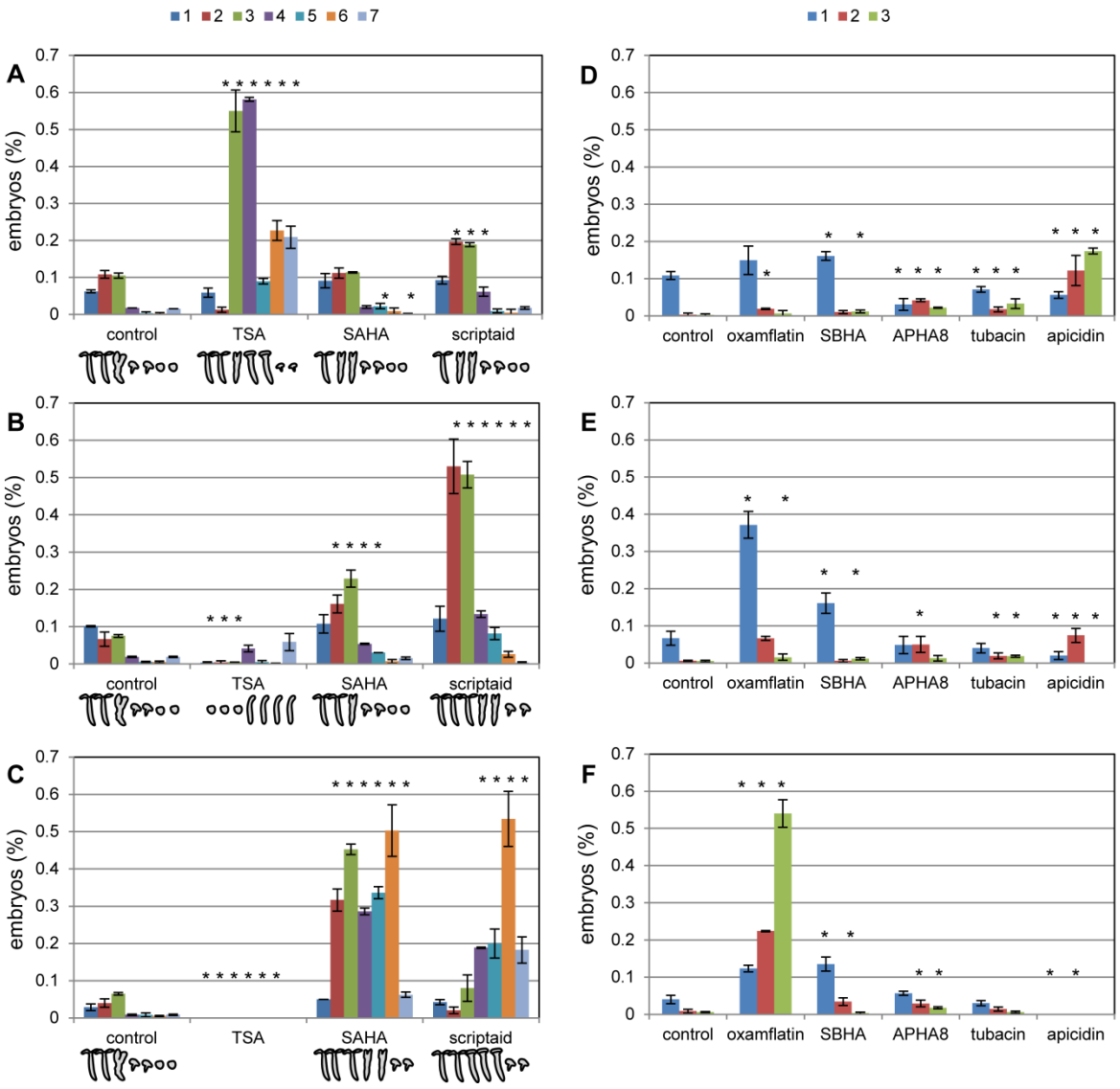

Figure 2. HDAC inhibitors increase embryo yield in *B. napus* microspore culture.

Embryo yield from control or HDAC inhibitor-treated DH12075 microspore cultures after 15 days of culture. The developmental stages of the starting material (1-7 or 1-3) are ranked from youngest to oldest, as described in Supplemental Data Set 1. The rankings are not absolute, but rather relative within one data set. Three concentrations of HDAC inhibitors were evaluated: 0.05 µM (**A** and **D**), 0.5 µM (**B** and **E**), 5 µM (**C** and **F**). *, statistically significant difference between the control and HDAC inhibitor treatment for the same developmental stage ($p < 0.05$; Student's *t* test).

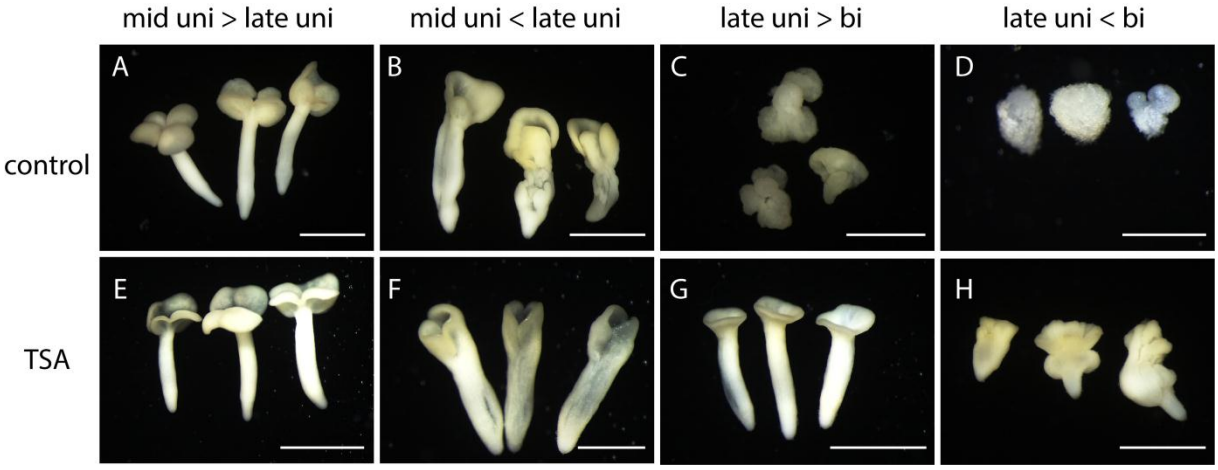

Figure 3. HDAC inhibitors improve embryo morphology in *B. napus*.

The morphology of embryos formed from microspore cultures started from progressively older stages of male gametophyte development (from left to right). The composition of each culture is shown in Supplemental Data Set 1. **(A-C)** Embryos from control cultures. Normal embryos (**A**), rough embryos (**B**), reduced embryos (**C**), ball-shaped embryos (**D**). **(E-H)** Embryos from 0.05 μM TSA-treated cultures. Normal embryos (**E**), wrinkled embryos (**F**), fused cotyledon embryos (**G**), reduced embryos (**H**). Scale bars, A, B, C, E, G, H: 2 mm; D, F: 1 mm.

HDACi-treated cultures, which did not form any shoots (Figure 4H, Supplemental Data Set 1). The overall conversion rate between control and HDACi treated embryos was similar, although differences were observed between the different embryo types in the different HDACi-treatments. For example, reduced embryos formed shoots in one treatment (5 μM scriptaid; Supplemental Data Set 1), while all other embryo types showed reduced germination in this treatment.

Our data indicate that HDACi treatment improved the morphology of embryos obtained from suboptimal stages of microspores/pollen. HDACi-treated do not show enhanced conversion rates compare to control embryos, but the total amount of functional embryos is increased by HDACi treatment.

Inhibition of HDAC activity improves embryo patterning

Next, we made thin sections of the different types of embryos found in control and HDACi-treated cultures to determine how inhibition of HDAC activity improves their morphology and conversion to plantlets. In control cultures, both the shoot apical meristem (SAM) and root apical meristem (RAM) of normal embryos were well developed (Figure 5A-C). The shoot apical meristem was characterized by a group of small cells with dense cytoplasm (Figure 5A and 5B), while the root apical meristem was characterized by a

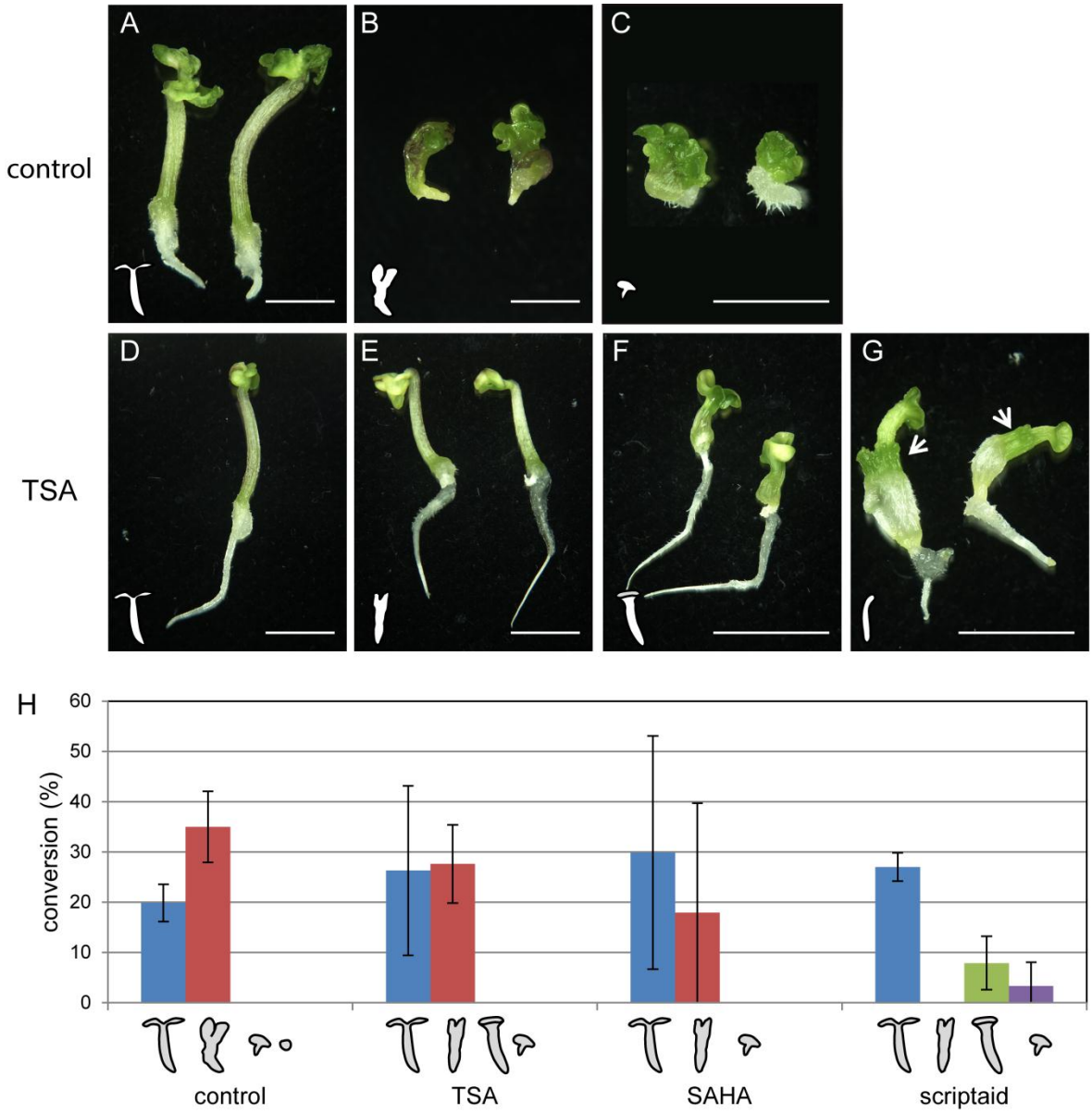

Figure 4. The effect of HDAC inhibitor treatment on embryo conversion in *B. napus*.

(A-G) Six-day old of seedlings from different types of embryos grown on germination medium. The embryo types are shown schematically in the image. **(A-C)** Germinated embryos from control cultures. These seedlings were derived from normal embryos (**A**), rough embryos (**B**), and reduced embryos (**C**). **(D-G)** Germinated embryos from TSA-treated cultures. These seedlings were derived from normal embryos (**D**), wrinkled embryos (**E**), fused cotyledon embryos (**F**), pin-shaped embryos (**G**). The arrow indicates somatic embryos on the hypocotyl. (**H**) The conversion rate of different embryo types after treatment of different starting microspore/pollen populations with different concentrations of HDAC inhibitors. The embryos from different stages of donor material were grouped for simplicity. The data is also available in Supplemental Data Set 1. Scale bars, 5 mm.

relatively well-organized stem cell niche adjacent to the provascular tissue. A few (1-3) quiescent centre (QC) cells could be recognized in the stem cell niche, which were surrounded by few small stem cells. These stem cells could be distinguished from other cells in the root tip by their small size, location (fifth layer from the root tip, at the border with the provascular tissue) and round shape (Figure 5D). The single provascular strand in the embryonic axis divided into two strands under the shoot meristem (Figure 5B) and extended into the two cotyledons. Rough embryos from control cultures had less well-defined epidermal and ground cell files, which contributed to their rough appearance, and a poorly defined SAM and RAM (Figure 5E-G), characterized by the absence of cytoplasmically-rich cells at the position of the SAM (Figure 5F) and lack of distinguishable QC cells in the root meristem (Figure 5H), although the provascular tissue was well-developed (Figure 5F). Reduced embryos from control cultures had poorly organized epidermal/ground cell files and poorly organized SAM, RAM and provascular tissue (as in rough embryos, Figure 5I-K). Ball-shaped embryos did not show any recognizable tissue organisation (Figure 5L).

The improved overall morphology of embryos from HDACi-treated cultures was reflected in the development of their cell files, meristems and apical-basal patterning. Unlike rough embryos from control cultures, the cell files of wrinkled embryos were well-organized, contributing to the reduced roughness of the embryo (Figure 5M, Q, U), although they were still less tightly packed compared to normal HDACi-treated embryos. The normal, wrinkled and fused cotyledon embryos developed a recognizable SAM (Figure 5N, R, V), RAM (Figure 5P, T, X) and provascular tissue (Figure 5N, R, V), although the SAM was reduced in size and less-well defined compared to the SAM of normal control embryos. Differences were observed between control and HDACi-treated embryos in the development of provascular tissue; two provascular strands were observed bifurcating the SAM in normal and rough control embryos, while only one provascular strand, which ended under the SAM, was observed in normal, wrinkled and fused cotyledon HDACi-treated embryos. In pin-shaped embryos from TSA-treated cultures, neither cotyledon primordia nor a SAM were observed at the apical end of the embryo (Figure 5Y-Z). Instead, the apical region was characterized by a dome-shaped structure containing small, uniformly-shaped cells subtended by larger cells (Figure 5Z). Pin-shaped embryos displayed normal provascular tissue development, but did not contain recognizable QC cells at the root tip (Figure 5Y, 5AA-AB).

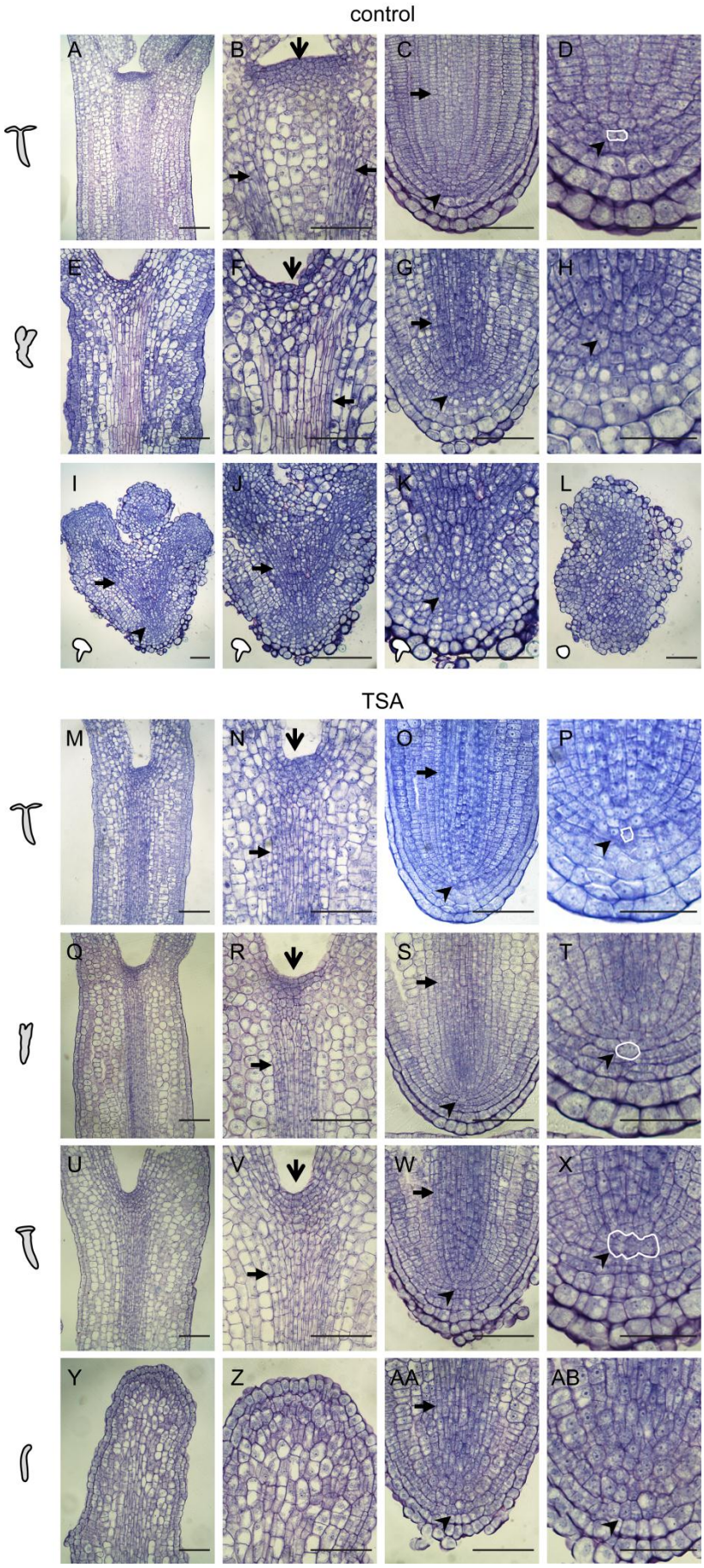

Figure 5. The effect of TSA on microspore embryo development in *B. napus.*

(A-L) Embryos from control cultures. Normal embryos (**A-D**), rough embryos (**E-H**), reduced embryos (**I-K**), ball-shaped embryos (**L**). The embryo types are shown schematically in the image. **(M-AB)** Embryos from TSA-treated cultures. Normal embryos (**M-P**), wrinkled embryos (**Q-T**), fused cotyledon embryos (**U-X**), pin-shaped embryos (**Y-AB**). **(A, E, M, Q, U, Y)** Shoot apex of embryos. **(B, F, N, R, V, Z)** Enlarged images of the shoot apex. **(C, G, J, K, O, S, W, AA)** Root apex of embryos. **(D, H, P, T, X, AB)** Enlarged images of the root apex. All the embryos were analysed at 20 days of culture. Open arrow, shoot apical meristem. Closed arrows, provascular tissue. Arrow head, stem cell niche of root apical meristem or normal position of the root apical meristem. Scale bars, A-C, E-G, I- K, M-O, Q-S, U-W, Y-AA, 100 µm. D, H, L, P, T, X, AB, 50 µm.

Our results indicate that HDACi treatment enhances the quality of embryos from suboptimal stages of donor gametophytes by improving the regularity of the cell files, axis elongation and cotyledon outgrowth.

Improved embryo patterning after HDACi treatment is reflected in embryo auxin response

A number of the the embryo phenotypes observed in control and HDACi-treated cultures (reduced, fused cotyledon, pin-shaped) resemble phenotypes observed in zygotic embryos treated with auxin transport inhibitors (Liu et al., 1993; Hadfi et al., 1998; Ramesar-Fortner and Yeung, 2006), or in other auxin-related processes (Mayer et al., 1993; Assaad et al., 1996; Friml et al., 2003; Furutani et al., 2004). Therefore, we examined whether auxin-related processes were altered in the embryos with abnormal morphology from both control and HDACi-treated cultures, using the *DR5:GFP* reporter as an indirect read-out of auxin levels and/or signalling (Ulmasov et al., 1997; Friml et al., 2003).

In *B. napus* zygotic embryos and normal microspore embryos, *DR5* expression is localized to the root pole at the late globular stage onwards (Figure 6A; Soriano et al., 2014/Chapter 5). *DR5* expression remains confined to the root pole as the embryo develops, but is not always observed at cotyledon tips, as described for Arabidopsis (Figure 6B, C; Soriano et al., 2014/Chapter 5; Benková et al., 2003). In abnormal embryos from control cultures, the *DR5* reporter was either expressed in fewer cells at the root tip (rough embryos; Figure 6D-F), or absent or only weakly expressed in one or two cells of the root tip (reduced embryos; Figure 6G). *DR5* expression was not observed in ball-shaped structures (not shown).

Normal embryos in HDACi-treated cultures showed a similar pattern of *DR5* expression as normal embryos from control cultures (Figure 6H-J). *DR5* expression was stronger and expressed more broadly in wrinkled embryos (Figure 6K-M), and in fused cotyledon

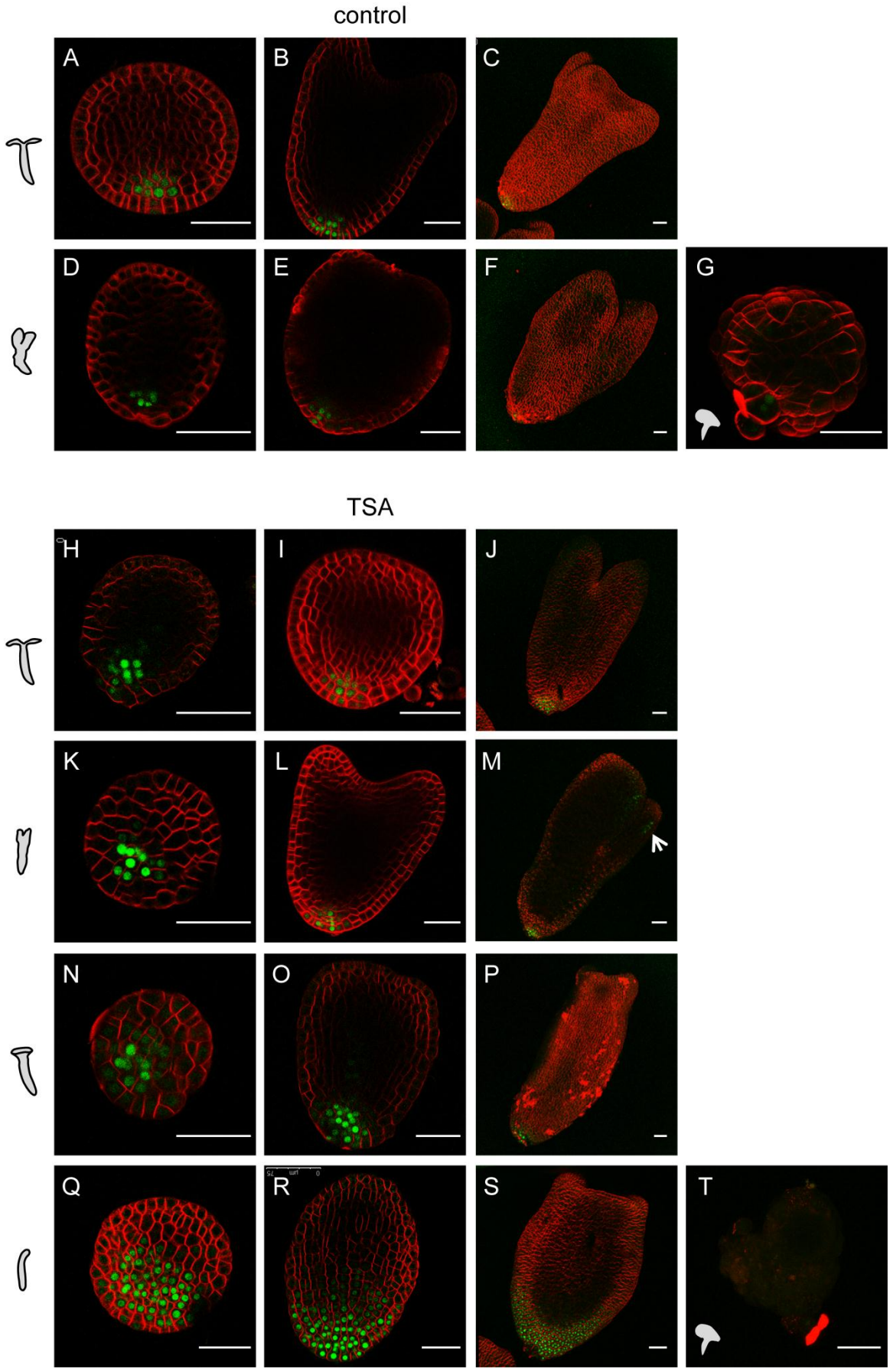

Figure 6. HDAC inhibition enhances auxin response in *B. napus* microspore embryos.

(A-G) *DR5:GFP* expression in embryos from control cultures. Normal embryos (**A-C**), rough embryos (**D-F**), reduced embryos (**G**). **(H-T)** *DR5:GFP* expression in embryos from TSA-treated cultures. Normal embryos (**H-J**), wrinkled embryos (**K-M**), fused cotyledon embryos (**N-P**), pin-shaped embryos (**Q-S**), reduced embryos (**T**). The embryo types are shown schematically in the image. Green, *GFP* signal; red, propidium iodide staining. Arrow, *DR5:GFP* expression in cotyledons. Scale bars, 50 µm.

(Figure 6N-P) and pin-shaped embryos (Figure 6Q-S), where *DR5* expression could also be observed in the provasculature (Figure 6O, R-S). *DR5* expression was increased and observed more frequently in the cotyledon tips of normal and wrinkled embryos from HDACi-treated cultures than those of control cultures. The reduced embryos from HDACi cultures did not or only weakly expressed *DR5*, as in the corresponding structures in control cultures (Figure 6G, T).

In general, inhibition of HDAC activity is associated with broader and stronger *DR5* expression, suggesting a role for enhanced basal auxin response in this process.

Discussion

Specificity for HDAC inhibitors

The HDACi TSA has been shown to be a potent enhancer of embryogenic cell division and embryo production in *B. napus* microspore culture (Li et al., 2014). Here we showed that additional compounds in the Rpd3/hda1/HD2 class of HDAC inhibitors also had a positive effect on embryo induction and/or yield in microspore culture for at least one of the concentrations tested. In general, TSA, scriptaid and apicidin were the most effective inducers of embryogenic cell division, and TSA, SAHA, scriptaid and oxamflatin were the most effective enhancers of embryo yield.

TSA and SAHA are considered pan-inhibitors of the mammalian Rpd3/hda1 class of HDACs, in that they inhibit all HDAC proteins in this family (Bieliauskas and Pflum, 2008). However Rpd3/hda1 HDAC isoforms also show differential sensitivity to different HDACi. For example, in mammals, HDAC8 is less sensitive to TSA, SAHA and scriptaid compared to other HDAC proteins (Blackwell et al., 2008; Khan et al., 2008). The isoform selectivity of HDACi may underlie the observed differences in the efficiency of embryo induction and yield of the HDACi that were tested in microspore culture. HDACi also show an optimal dosage for HDAC inhibition. For example, TSA inhibits the *in vitro* activity of different mammalian HDAC isoforms in the nM range, while SAHA, scriptaid, and oxamflatin are most effective in the µM

range (Blackwell et al., 2008). The dosage curve for TSA in microspore culture is in the nanomolar range (data not shown), but the dosage curves for the other HDACi has not been rigorously examined. Micromolar concentrations of SAHA, scriptaid and oxamflatin were needed to obtain similar efficiencies as nanomolar concentrations TSA, suggesting that the optimum dose for these compounds is higher than tested here.

Embryogenic callus

Treatment with HDACi induces massive induction of embryogenic cells in microspore culture, and although embryo yield is also increased after HDACi treatment, the vast majority of embryogenic cells fail to form embryos. Instead, these cells form clusters of loosely connected cells that divide slowly and eventually die. Cell loosening can already be observed after one to two cell divisions (Li et al., 2014; Soriano et al., 2014/Chapter 5), indicating that this embryogenic callus pathway is induced early in culture. These callus-like structures are also observed in control cultures, although in lower numbers, suggesting that their formation is a normal response to the induction treatment. Understanding why these callus-like structures fail to differentiate can be instrumental in designing strategies to improve their differentiation to functional embryos. Callus-like structures do not express the auxin response marker *DR5*, whereas normal embryos do (Soriano et al., 2014/Chapter 5), suggesting a link between the failure to mount an early auxin response and histodifferentiation.

The major change in gene expression between globular embryos and embryogenic callus was the misregulation of key developmental regulators, especially those involved in patterning and in auxin-related processes. Misregulation of these genes might simply be a downstream effect that marks the lack of tissue differentiation in these structures. TSA-induced callus-like structures lack apical and basal polarity, thus genes that might normally be apically or basally expressed in normal embryos might be weakly or not expressed or spatially mis-expressed in embryogenic callus. In support of this, *PIN* gene expression is misregulated, but polar auxin transport is not required for apical-basal patterning and epidermal specification in microspore embryos (Soriano et al., 2014/Chapter 5). Alternatively, misexpression of these patterning genes might reflect the deregulation of key upstream regulatory factors that determine the identity of the different embryonic tissues.

HDACs regulate auxin signalling

We observed that haploid embryo morphology is negatively influenced by progressively older stages of donor gametophytes. In control cultures, a continuum of developmental phenotypes was observed, from normal → rough → reduced → ball shaped embryos, which correlated with the increasing developmental age of the donor pollen (Figure 7A). Treating microspore cultures with HDACi improved the apical-basal pattern of embryos from progressively older microspore/pollen populations in a dose and HDACi-specific manner, but also had a negative effect on apical pole development, by reducing the quality of the shoot meristem and eventually cotyledon outgrowth (Figure 7B). Treatment with higher concentrations of TSA and scriptaid had a strong effect on embryo development, either by abolishing embryo development completely or by inducing fused cotyledon and/or pin-shaped embryos. The continuum of developmental phenotypes after HDACi treatment can therefore be described as a transition from apical-basal compromised embryos (ball-shaped) to basally hyperactivated embryos (pin-shaped) i.e. ball shaped → reduced → wrinkled/rough → normal → fused cotyledon →pin-shaped (Figure 7B). Together the data suggest that there is a continuum of embryo morphologies, from ball-shaped to pin-shaped, that develop in microspore culture, that results from changes in the histone acetylation status of the cell and increased basal auxin response.

HDACi-treated embryos showed a stronger and broader basal *DR5* auxin response than control embryos, regardless of their origin and phenotype. In Arabidopsis zygotic embryos, auxin maxima are formed in the uppermost cell of the embryonic suspensor, the hypophysis (the precursor of the root meristem) and in the tips of the cotyledon primordia. These maxima are established in part by directional PIN-mediated auxin efflux. PIN expression is first observed after zygote division, when PIN7 becomes expressed at the apical side of the basal cell, driving auxin flow from the suspensor to the embryo proper and *DR5* expression in the embryo proper. PIN1 is expressed in an apolar fashion in the 8-celled embryo proper stage, while *PIN7* expression remains at the apical side of the suspensor. PIN1 polarity changes at the globular stage, where it is expressed apically in the protoderm and basally in the provascular tissue. At the same time, *PIN7* expression switches from the apical to basal side of the suspensor and is replaced in the hypophysis by *PIN4* expression. The net effect is that auxin accumulates in the hypophysis, and at the tips of the cotyledon promordia. HDACi treatment enhanced basal *DR5* expression suggesting that basal embryo identity is

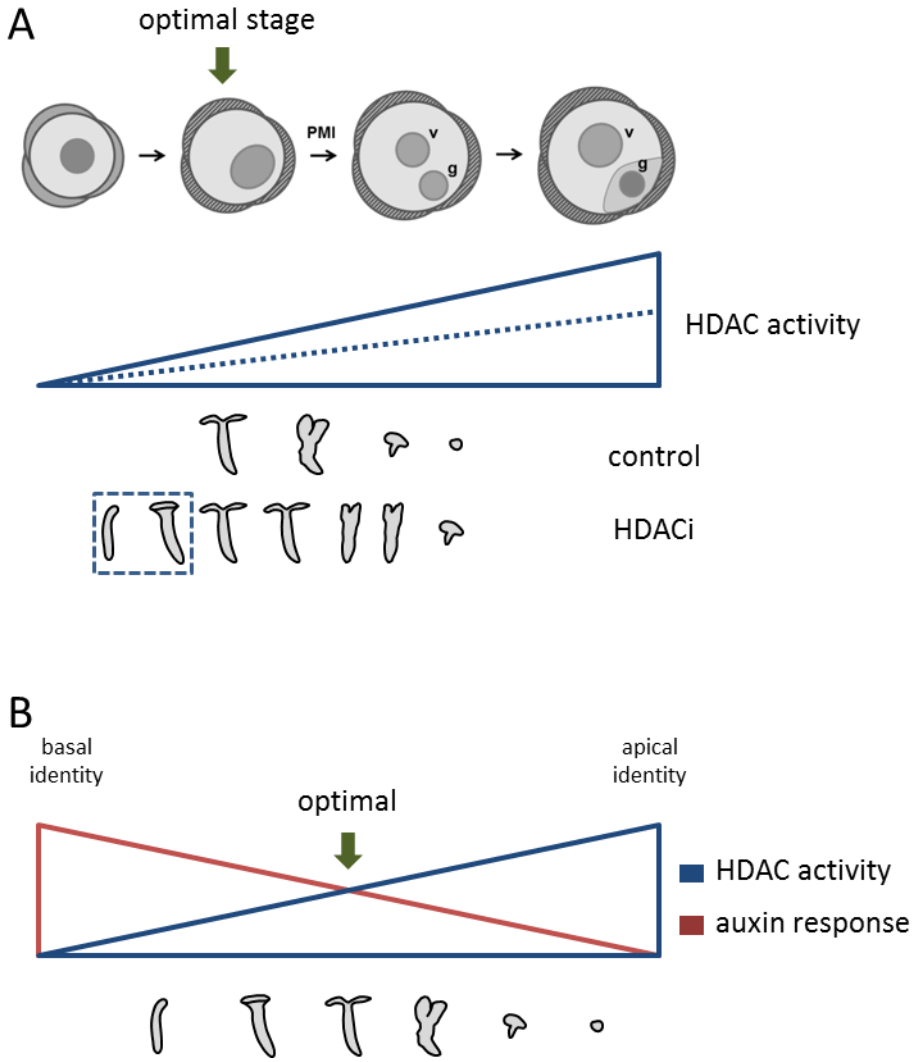

Figure 7. Model for the role of histone deacetylation in microspore embryo patterning.

(**A**) Progressively older stages of microspore/pollen development are characterized by increased HDAC activity (solid triangle), which has a negative effect on embryo patterning. Treatment with HDAC inhibitors (dotted lines) reduces HDAC activity, allowing better patterning at suboptimal stages. Note that treatment with higher concentrations of TSA or scriptaid negatively affects apical embryo patterning (square box), but in a stage independent manner.

(**B**) HDAC proteins repress auxin response. A relatively low HDAC activity and a relatively high auxin response enforces basal embryo identity, while a relatively high HDAC activity and a relatively low auxin response enforces apical identity. When the two processes are in balance, then apical and basal patterning is also balanced. Embryos from progressively older stages of microspores/pollen show reduced basal identity (A), which can be complemented by treatment with HDAC inhibitors; however basal patterning predominates at the expense of apical growth (e.g. in pin- and fused cotyledon embryos) when HDAC activity is too strongly repressed. The model does not take into account the HDAC specificity or dosage-dependent activities of the different HDAC inhibitors.

strengthened by HDACi treatment. Initially, this stronger basal identity is balanced in that apical growth is also promoted, and the embryo axis elongates and the cotyledons expand. Application of TSA and scriptaid at certain concentrations appears to have a much stronger effect on basal identity, so that apical development in the most affected embryos (fused cotyledon and pin-shaped) is compromised. The basal embryo identity in these embryos might be so strong that auxin does not flow back up into cotyledon via epidermal PIN1, leading to loss of auxin at the apical pole. A local auxin gradient is required for cotyledon formation (Benková et al., 2003), thus a lower amount of auxin in the apical pole might reduce expression of the *CUC* boundary genes (leading to fused cotyledon embryos) and/or fall below the threshold required for cotyledon initiation (leading to pin-shaped embryos). Alternatively, the apical pole of the embryo may take on a basal identity, as described for the *topless* mutant (Long et al., 2002).

Auxin has been shown to regulate gene expression during plant development by altering the acetylation status of histones (Anzola et al., 2010; Manzano et al., 2012). TOPLESS (TPL), a Groucho (Gro)/Tup1 type transcriptional co-repressor controls auxin-mediated embryo patterning through interaction with the Rpd3/Class 1 HDAC, HDA19 (Long et al., 2006). TPL is required at the globular-to-heart transition stage to repress basal identity in the apical half of the embryo, allowing shoot growth (Long et al., 2002). The *tpl-1* mutant carries a dominant negative, temperature sensitive mutation that interferes with the function of four other functionally-redundant TOPLESS-RELATED (TPR) proteins (Long et al., 2006). Homozygous *tpl-1* mutants show either loss of apical structures (24 ^{o}C) or conversion of the apical pole into a root (29 ^{o}C). Loss-of-function *hda19* mutants show only mild changes in cotyledon morphology at 24 ^{o}C, but at 29 ^{o}C, *hda19* mutants phenocopy *tpl* phenotypes, indicating that HDA19 and TPL function together to control basal cell fate establishment in the Arabidopsis embryo (Long et al., 2006). TPL was shown to interact directly with the EAR-domain of AUX/IAA transcriptional co-repressor IAA12/BODENLOS (BDL), which inhibits transcription of the auxin response factor ARF5/MONOPTEROS (MP) (Szemenyei et al., 2008). The single *bdl* loss-of-function mutant does not show a phenotype, but enhances the *tpl* phenotype in the *tpl* mutant background. These results demonstrate that TPL controls ARF expression in an auxin-dependent manner. Interestingly, *DR5* expression did not show ectopic apical expression in the *tpl* mutant, which might reflect a temporal delay associated

with reprogramming from shoot to root identity or the fact that *DR5* is an artificial reporter (Osmont and Hardtke, 2008).

The changes in microspore embryo morphology observed after HDACi treatment can be explained in light of the effect of TPL-BDL-HDAC repression on ARF expression/auxin response (Figure 7). In this scenario, HDACi treatment inactivates HDAC activity, leading to loss of or reduced ARF repression/auxin response by the TPL-AUX/IAA corepressors. The enhanced basal identity induced by enhanced ARF expression/auxin response initially leads to outgrowth and patterning of the apical and basal poles, but when ARF expression/auxin response is hyperactivated it leads to overdevelopment of the basal pole at the expense of the apical pole. Whether the apical pole is converted to a basal pole, as in *tpl/hda19* mutants, or loses its apical identity due to reduced auxin flow from the over-developed basal pole remains to be determined.

The progressive changes in apical-basal patterning observed in embryos derived from progressively older stages of donor male gametophytes raises the possibility that these suboptimal microspores/pollen stages are associated with an inherently reduced auxin response (Figure 7) that can be rescued by increased histone acetylation.

The HDACi used in our experiments were only applied to microspore cultures for 20 hours, together with the heat stress treatment, and then removed by refreshing the culture medium. Histone acetylation and deacetylation are highly dynamic processes (Chen and Tian, 2007; Wang et al., 2009), and treatment with HDACi can be reversed by removing the inhibitor from the medium (Waterborg, 1998). For example, the aberrant root cell patterning induced by TSA treatment can be reversed by removing TSA, and conversely, continuous TSA-treatment is required for maintaining this aberrant root cell patterning (Xu et al., 2005). The dynamic nature of the histone acetylation-deacetylation cycle and the reversible nature of HDACi treatment suggest that transient treatment of microspores and pollen with HDACi induces developmental changes that have long lasting effects on their development.

Materials and methods

Plant material and culture

Brassica napus L. cv. Topas DH4079 and DH12075 were used as donor plants for microspore embryo culture. The *B. napus* plant growth and microspore isolation procedures

were performed as described previously (Custers, 2003; Li et al., 2014; Soriano et al; 2014/Chapter 5).

Chemical treatments

All HDACis (Sigma-Aldrich) were prepared as 1 mM stock solutions dissolved in dimethyl sulfoxide (DMSO) and stored at -20 ^{o}C. The list of tested HDACis is shown in Supplemental Table I. Freshly isolated microspores were resuspended in NLN-13 medium containing the appropriate HDACi or the same volume of DMSO as a control, and cultured for 20 hours at 33^{o}C. After this period the cultures were centrifuged at 200 g for 3 min, resuspended in fresh NLN-13 medium without HDACis, and transferred to 25 ^{o}C. Cultures were incubated in the dark. A minimum of three independent experiments were conducted with two biological replicates and three technical replicates.

Microarray analysis

Three independent microspore cultures from DH4079 were cultured at 32 ^{o}C for 20 hours with either TSA (Sigma-Aldrich) or DMSO and then transferred to 25 ^{o}C. The samples were harvested after seven days days of culture by filtration through 70 µm nylon screens. Cell clusters that did not pass through the screen were collected for RNA isolation. Total RNA isolation and on-column DNase digestion were performed using the InviTrap Spin Plant RNA Mini Kit (Invitek) according to the manufacturer's instructions. One microgram of total RNA from each sample was sent to the NASC Affymetrix Service (http://affymetrix.arabidopsis.info/) for hybridisation to the Affymetrix Brassica Exon 1.0 ST GeneChip. Probe annotations were downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The identifier for the annotation is GPL10733.

The expression data was subjected to normalization using the RMA method from the 'Affy' Bioconductor package. Log2-transformed expression values were identified as differentially expressed using a Student's *t*-test. Multiple hypothesis testing correction was done using the Holm's method (Holm, 1979) implemented in the multtest's Bioconductor package. Mapman (Thimm et al., 2004) was used to identify functional categories of differentially-expressed genes. Gene Ontology (GO) analysis was performed with BiNGO (Maere et al., 2005). First Brassica unigenes were associated to Arabidopsis genes by sequence similarity using the file "BrasEx1s.unigene_v_at.1e-5.tophit.txt" downloaded from

http://www.brassica.info/resource/transcriptomics. Next, the associated Arabidopsis genes were used to calculate GO enrichment for the differentially expressed Brassica unigenes compared to all the Brassica unigenes represented on the microarrays. A probe-set was considered to be differentially expressed when the FDR was <0.05 (Holm, 1979) and the abs (logRatio) >0.7.

The quantitative RT-PCR primers for microarray validation were designed based on oligonucleotide probes from Affymetrix GeneChip® Brassica Exon 1.0ST Array (Supplemental Data Set 2, Love et al., 2010).

Cytological and morphological analyses

Microspore-derived embryos for histological analysis were collect at 20 days of culture and fixed in 70% ethanol: acetic acid (3:1) for 24h at 4 ^{o}C and then stored in 70% ethanol. The embryos were dehydrated gradually (70%, 80% and 100% ethanol, 15 min each), then embedded in Technovit 7100 (Fisher Scientific) according to the manufacturer's instructions. Ten micron thick longitudinal serial sections with were cut using a Leica Reichert-Jung 2040 autocut microtome, stained with 0.05% Toluidine Blue for 3 minutes, then mounted in euparol after drying and put on cover glass, followed by observed under a Zeiss Axioskop epifluorescence microscope.

The developmental stage of cells in microspore culture was determined using the nuclear stain 4', 6-diamidino-2-phenylindole (DAPI, 1.25 μg/ml) according to Custers (2003), visualised using a Zeiss Axioskop epifluorescence microscope (excitation wavelength, 400 nm; emission wavelength, 420 nm). Approximately two hundred microspores or multicellular clusters were counted for each sample. GFP was imaged using confocal laser scanning microscopy (CLSM; Leica DM5500 Q). The GFP was excited with an argon laser line at 488 nm and detected with a 505–530 nm emission filter. Samples were stained with propidium iodide (10 mg/ml; Sigma-Aldrich). Propidium iodide and red autofluorescence were excited at 532 nm and detected with a 620-660 nm emission filter. The optical slices were median filtered with Leica LAS AF software. The *DR5::GFP* (*GIIK DR5rev::SV40:3GFP*) construct was transformed to *Agrobacterium tumefaciens* strain C58C1 pMP90 and then to *B. napus* DH12075 as described previously (Weijers et al., 2006; Soriano et al., 2014/Chapter 5).

Acknowledgements

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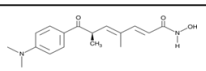
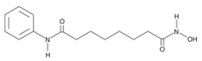
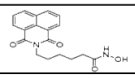
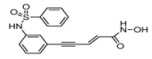
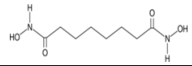
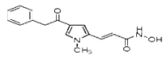
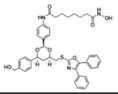
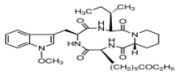
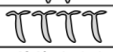
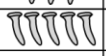
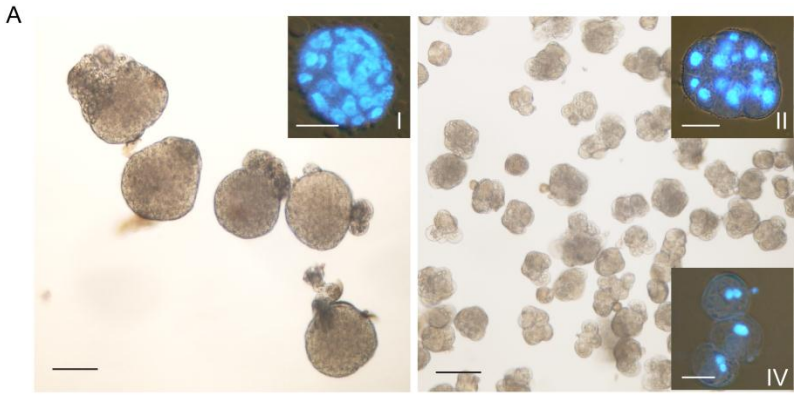
Supplemental data

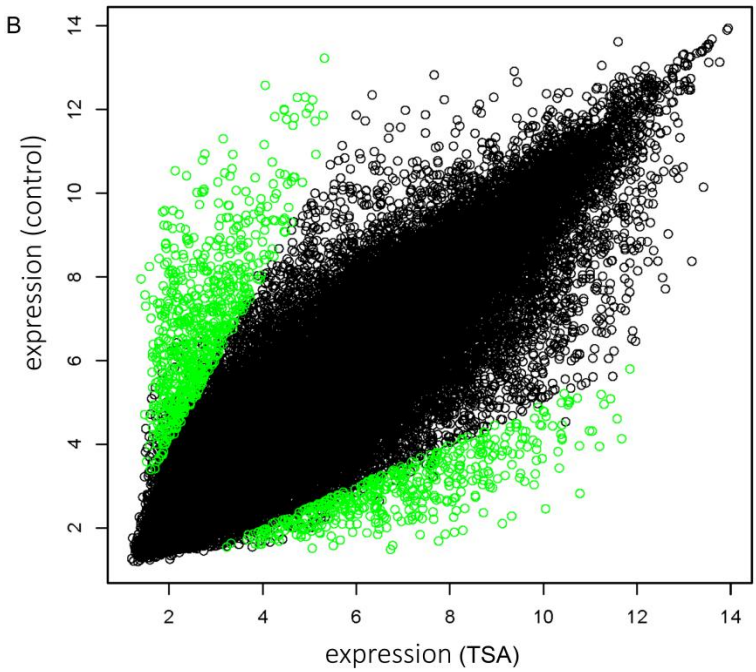
Supplemental Table I. List of HDAC inhibitors used in this study

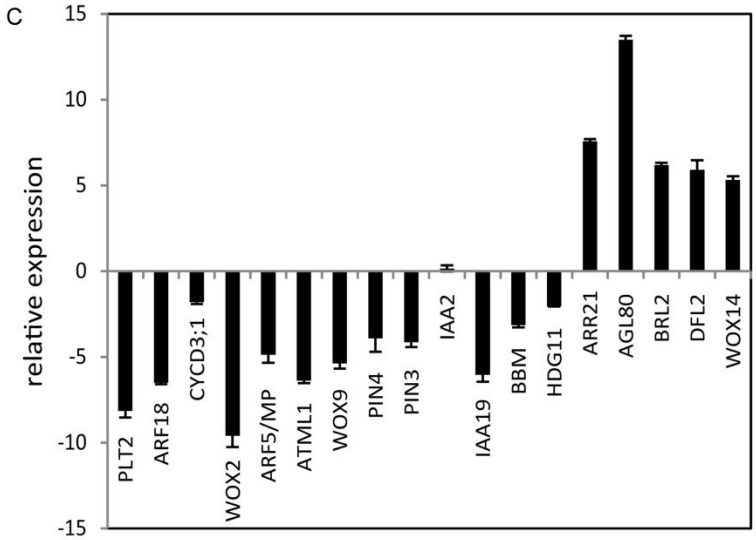
	Inhibitor	structure	group
1	TSA		hydroxamate
2	SAHA		hydroxamate
3	scriptaid		hydroxamate
4	oxamflatin		hydroxamate
5	SBHA		hydroxamate
6	APHA 8		hydroxamate
7	tubacin		hydroxamate
8	apicidin		cyclic tetrapeptide

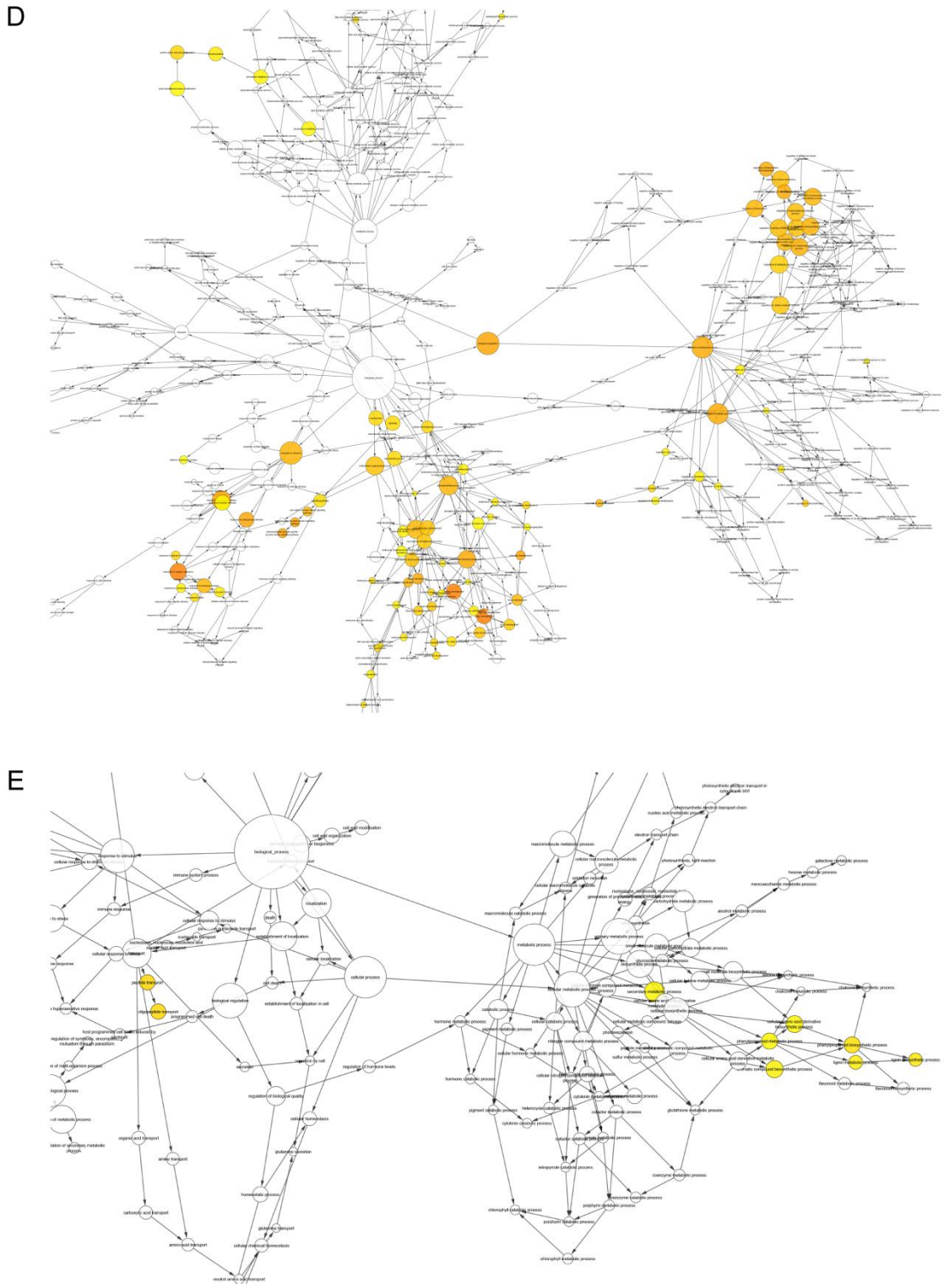
Supplemental Table II. The effect of HDAC inhibitors on *B. napus* embryo development

compounds	concentration (μM)	embryo morphology			
		Initial gametophyte composition			
		mid uni > late uni	mid uni < late uni	late uni > bi	late uni < bi
control					
SAHA	0.05				
	0.5				
	5				
TSA	0.05				
	0.5	---	---		
	5	---	---	---	---
scriptaid	0.05				
	0.5				
	5				


A
I
II
IV


B
expression (control)
expression (TSA)
2
4
6
8
10
12
14


C
relative expression
15
10
5
0
-5
-10
-15
PLT2
ARF18
CYCD3;1
WOX2
ARF5/MP
ATML1
WOX9
PIN4
PIN3
IAA2
IAA19
BBM
HDG11
ARR21
AGL80
BRL2
DFL2
WOX14



Supplemental Figure 1. Gene expression analysis of embryogenic cell types in microspore culture: analysis and distribution of functional categories of the differentially expressed probes.

(A) Samples from seven day-old microspore cultures used for microarray analysis. Globular stage embryos (left) and Type III and IV embryogenic callus (right). The insets show DAPI-stained embryos. Scale bar, 100 µm. Inset, scale bar, 50 µm. **(B)** Scatter plot of significantly expressed genes for TSA-treated cultures and control cultures. **(C)** Validation of microarray gene expression data by quantitative real-time RT-PCR. Independent *B. napus* microspore cultures were collected under the same conditions used for the microarray analysis. The relative

expression of each gene was calculated according to Livak and Schmittgen (2001) using the corresponding mock treatment as the calibrator and the SAND gene as the reference. Relative expression is shown as log2 values (- ΔΔCt). **(D)** Overrepresented Gene Ontology functional categories for TSA down-regulated genes. Significantly overrepresented nodes are shaded yellow, with increased shading at more significant *p*-values. (**E)** Overrepresented Gene Ontology functional categories for TSA up-regulated genes. Significantly overrepresented nodes are shaded yellow, with increased shading at more significant *p*-values.

References

Abe, M., Takahashi, T., and Komeda, Y. (1999). Cloning and characterization of an L1 layer-specific gene in *Arabidopsis thaliana*. Plant Cell Physiol. **40:** 571-580.

Abe, M., Katsumata, H., Komeda, Y., and Takahashi, T. (2003). Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*. Development **130:** 635-643.

Alinsug, M.V., Yu, C.-W., and Wu, K. (2009). Phylogenetic analysis, subcellular localization, and expression patterns of RPD3/HDA1 family histone deacetylases in plants. BMC Plant Biol. **9:** 37.

Anzola, J.M., Sieberer, T., Ortbauer, M., Butt, H., Korbei, B., Weinhofer, I., Müllner, A.E., and Luschnig, C. (2010). Putative *Arabidopsis* transcriptional adaptor protein (PROPORZ1) is required to modulate histone acetylation in response to auxin. Proc. Natl. Acad. Sci. USA **107:** 10308-10313.

Assaad, F., Mayer, U., Wanner, G., and Jürgens, G. (1996). The *KEULE* gene is involved in cytokinesis in *Arabidopsis*. Mol. Gen. Genet. **253:** 267-277.

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. Plant Cell **23:** 69-80.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115:** 591-602.

Berleth, T., and Jurgens, G. (1993). The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. Development **118:** 575-587.

Bertrand, P. (2010). Inside HDAC with HDAC inhibitors. Eur. J. Med. Chem. **45:** 2095-2116.

Bieliauskas, A.V., and Pflum, M.K.H. (2008). Isoform-selective histone deacetylase inhibitors. Chem. Soc. Rev. **37:** 1402-1413.

Blackwell, L., Norris, J., Suto, C.M., and Janzen, W.P. (2008). The use of diversity profiling to characterize chemical modulators of the histone deacetylases. Life Sci. **82:** 1050-1058.

Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A.A., and Miki, B.L. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell **14:** 1737-1749.

Chandler, J.W., Cole, M., Flier, A., Grewe, B., and Werr, W. (2007). The AP2 transcription factors DORNRÖSCHEN and DORNRÖSCHEN-LIKE redundantly control *Arabidopsis* embryo patterning via interaction with PHAVOLUTA. Development **134:** 1653-1662.

Chandler, J.W., Jacobs, B., Cole, M., Comelli, P., and Werr, W. (2011). *DORNRÖSCHEN-LIKE* expression marks *Arabidopsis* floral organ founder cells and precedes auxin response maxima. Plant Mol. Biol. **76:** 171-185.

Chen, M.-K., Wilson, R.L., Palme, K., Ditengou, F.A., and Shpak, E.D. (2013). *ERECTA* family genes regulate auxin transport in the shoot apical meristem and forming leaf primordia. Plant Physiol. **162:** 1978-1991.

Chen, Z.J., and Tian, L. (2007). Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochim. Biophys. Acta - Gene Struct. Expression **1769:** 295-307.

Cigliano, R.A., Cremona, G., Paparo, R., Termolino, P., Perrella, G., Gutzat, R., Consiglio, M.F., and Conicella, C. (2013). Histone deacetylase AtHDA7 is required for female gametophyte and embryo development in *Arabidopsis*. Plant Physiol. **163:** 431-440.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. Development **121:** 2057-2067.

Custers, J.B.M. (2003). Microspore culture in rapeseed (*Brassica napus* L.). In Doubled haploid production in crop plants: a manual, M. Maluszynski, K.J. Kasha, B.P. Forster, and I. Szarejko, eds (Dordrecht: Kluwer Academic Publishers), pp. 185-193.

Earley, K., Lawrence, R.J., Pontes, O., Reuther, R., Enciso, A.J., Silva, M., Neves, N., Gross, M., Viegas, W., and Pikaard, C.S. (2006). Erasure of histone acetylation by *Arabidopsis* HDA6 mediates large-scale gene silencing in nucleolar dominance. Genes Dev. **20:** 1283-1293.

Earley, K.W., Pontvianne, F., Wierzbicki, A.T., Blevins, T., Tucker, S., Costa-Nunes, P., Pontes, O., and Pikaard, C.S. (2010). Mechanisms of HDA6-mediated rRNA gene silencing: suppression of intergenic Pol II transcription and differential effects on maintenance versus siRNA-directed cytosine methylation. Genes Dev. **24:** 1119-1132.

Ferrie, A., Epp, D., and Keller, W. (1995). Evaluation of *Brassica rapa L.* genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Rep. **14:** 580-584.

Ferrie, A.M., and Möllers, C. (2011). Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss. Org. **104:** 375-386.

Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R., and Pavletich, N.P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature **401:** 188-193.

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

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical–basal axis of *Arabidopsis*. Nature **426:** 147-153.

Furuta, K., Kubo, M., Sano, K., Demura, T., Fukuda, H., Liu, Y.-G., Shibata, D., and Kakimoto, T. (2011). The CKH2/PKL chromatin remodeling factor negatively regulates cytokinin responses in Arabidopsis calli. Plant Cell Physiol. **52:** 618-628.

Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M., and Aida, M. (2004). *PIN-FORMED1* and *PINOID* regulate boundary formation and cotyledon development in *Arabidopsis* embryogenesis. Development **131:** 5021-5030.

Galinha, C., Hofhuis, H., Luijten, M., Willemsen, V., Blilou, I., Heidstra, R., and Scheres, B. (2007). PLETHORA proteins as dose-dependent master regulators of *Arabidopsis* root development. Nature **449:** 1053-1057.

Germanà, M.A. (2011). Anther culture for haploid and doubled haploid production. Plant Cell Tiss. Org. **104:** 283-300.

Grozinger, C.M., and Schreiber, S.L. (2002). Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem. Biol. **9:** 3-16.

Hadfi, K., Speth, V., and Neuhaus, G. (1998). Auxin-induced developmental patterns in *Brassica juncea* embryos. Development **125:** 879-887.

Haecker, A., Groß-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M., and Laux, T. (2004). Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development **131:** 657-668.

Hassan, H., Scheres, B., and Blilou, I. (2010). JACKDAW controls epidermal patterning in the *Arabidopsis* root meristem through a non-cell-autonomous mechanism. Development **137:** 1523-1529.

Hollender, C., and Liu, Z. (2008). Histone deacetylase genes in *Arabidopsis* development. J. Integr. Plant Biol. **50:** 875-885.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. Scand. J. Stat. **6:** 65-70.

Huang, L., Sun, Q., Qin, F., Li, C., Zhao, Y., and Zhou, D.-X. (2007). Down-regulation of a *SILENT INFORMATION REGULATOR2*-related histone deacetylase gene, *OsSRT1*, induces DNA fragmentation and cell death in rice. Plant Physiol. **144:** 1508-1519.

Khan, N., Jeffers, M., Kumar, S., Hackett, C., Boldog, F., Khramtsov, N., Qian, X., Mills, E., Berghs, S., and Carey, N. (2008). Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. Biochem. J **409:** 581-589.

Kim, K.-C., Lai, Z., Fan, B., and Chen, Z. (2008). *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell **20:** 2357-2371.

Kim, W., Latrasse, D., Servet, C., and Zhou, D.-X. (2013). Arabidopsis histone deacetylase HDA9 regulates flowering time through repression of AGL19. Biochem. Biophys. Res. Commun. **432:** 394-398.

Kwak, S.-H., and Schiefelbein, J. (2007). The role of the SCRAMBLED receptor-like kinase in patterning the *Arabidopsis* root epidermis. Dev. Biol. **302:** 118-131.

Li, H., Soriano, M., Cordewener, J., Muiño, J.M., Riksen, T., Fukuoka, H., Angenent, G.C., and Boutilier, K. (2014). The histone deacetylase inhibitor trichostatin A promotes totipotency in the male gametophyte. Plant Cell**:** tpc. 113.116491.

Li, Y., Butenko, Y., and Grafi, G. (2005). Histone deacetylation is required for progression through mitosis in tobacco cells. Plant J. **41:** 346-352.

Liu, C., Xu, Z., and Chua, N.-H. (1993). Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell **5:** 621-630.

Liu, X., Yu, C.-W., Duan, J., Luo, M., Wang, K., Tian, G., Cui, Y., and Wu, K. (2012). HDA6 directly interacts with DNA methyltransferase MET1 and maintains transposable element silencing in *Arabidopsis*. Plant Physiol. **158:** 119-129.

Liu, X., Chen, C.-Y., Wang, K.-C., Luo, M., Tai, R., Yuan, L., Zhao, M., Yang, S., Tian, G., and Cui, Y. (2013). PHYTOCHROME INTERACTING FACTOR3 associates with the histone deacetylase HDA15 in repression of chlorophyll biosynthesis and photosynthesis in etiolated *Arabidopsis* seedlings. Plant Cell **25:** 1258-1273.

Long, J.A., Ohno, C., Smith, Z.R., and Meyerowitz, E.M. (2006). TOPLESS regulates apical embryonic fate in *Arabidopsis*. Science **312:** 1520-1523.

Long, J.A., Woody, S., Poethig, S., Meyerowitz, E.M., and Barton, M.K. (2002). Transformation of shoots into roots in *Arabidopsis* embryos mutant at the *TOPLESS* locus. Development **129:** 2797-2806.

Love, C.G., Graham, N.S., Lochlainn, S.Ó., Bowen, H.C., May, S.T., White, P.J., Broadley, M.R., Hammond, J.P., and King, G.J. (2010). A Brassica exon array for whole-transcript gene expression profiling. PloS one **5:** e12812.

Luo, M., Wang, Y.-Y., Liu, X., Yang, S., Lu, Q., Cui, Y., and Wu, K. (2012a). HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*. J. Exp. Bot. **63:** 3297-3306.

Luo, M., Yu, C.-W., Chen, F.-F., Zhao, L., Tian, G., Liu, X., Cui, Y., Yang, J.-Y., and Wu, K. (2012b). Histone deacetylase HDA6 is functionally associated with AS1 in repression of *KNOX* genes in *Arabidopsis*. PLoS Genet. **8:** e1003114.

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature **453:** 1094-1097.

Maere, S., Heymans, K., and Kuiper, M. (2005). BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics **21:** 3448-3449.

Manzano, C., Ramirez-Parra, E., Casimiro, I., Otero, S., Desvoyes, B., De Rybel, B., Beeckman, T., Casero, P., Gutierrez, C., and del Pozo, J.C. (2012). Auxin and epigenetic regulation of *SKP2B*, an F-box that represses lateral root formation. Plant Physiol. **160:** 749-762.

Matsumoto, N., and Okada, K. (2001). A homeobox gene, *PRESSED FLOWER*, regulates lateral axis-dependent development of *Arabidopsis* flowers. Genes Dev. **15:** 3355-3364.

Mayer, U., Buttner, G., and Jurgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development **117:** 149-162.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001). Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. Nature **411:** 709-713.

Millar, C.B., and Grunstein, M. (2006). Genome-wide patterns of histone modifications in yeast. Nat. Rev. Mol. Cell Biol. **7:** 657-666.

Miller, T.A., Witter, D.J., and Belvedere, S. (2003). Histone deacetylase inhibitors. J. Med. Chem. **46:** 5097-5116.

Murphy, J.P., McAleer, J.P., Uglialoro, A., Papile, J., Weniger, J., Bethelmie, F., and Tramontano, W.A. (2000). Histone deacetylase inhibitors and cell proliferation in pea root meristems. Phytochemistry **55:** 11-18.

Nakamura, M., Katsumata, H., Abe, M., Yabe, N., Komeda, Y., Yamamoto, K.T., and Takahashi, T. (2006). Characterization of the class IV homeodomain-leucine zipper gene family in *Arabidopsis*. Plant Physiol. **141:** 1363-1375.

Nawy, T., Bayer, M., Mravec, J., Friml, J., Birnbaum, K.D., and Lukowitz, W. (2010). The GATA factor *HANABA TARANU* is required to position the proembryo boundary in the early *Arabidopsis* Embryo. Dev. Cell **19:** 103-113.

Nguyen, H.N., Kim, J.H., Jeong, C.Y., Hong, S.-W., and Lee, H. (2013). Inhibition of histone deacetylation alters Arabidopsis root growth in response to auxin via PIN1 degradation. Plant Cell Rep. **32:** 1625-1636.

Nogué, F., Grandjean, O., Craig, S., Dennis, S., and Chaudhury, M. (2000). Higher levels of cell proliferation rate and cyclin *CycD3* expression in the *Arabidopsis amp1* mutant. Plant Growth Regul. **32:** 275-283.

Osmont, K.S., and Hardtke, C.S. (2008). The *topless* plant developmental phenotype explained. Genome Biol. **9:** 219.

Overvoorde, P.J., Okushima, Y., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Liu, A., Onodera, C., and Quach, H. (2005). Functional genomic analysis of the *AUXIN/INDOLE-3-ACETIC ACID* gene family members in *Arabidopsis thaliana*. Plant Cell **17:** 3282-3300.

Pandey, R., MuÈller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W., and Jorgensen, R.A. (2002). Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res. **30:** 5036-5055.

Rademacher, E.H., Möller, B., Lokerse, A.S., Llavata‐Peris, C.I., van den Berg, W., and Weijers, D. (2011). A cellular expression map of the Arabidopsis *AUXIN RESPONSE FACTOR* gene family. Plant J. **68:** 597-606.

Ramesar-Fortner, N.S., and Yeung, E.C. (2006). Physiological influences in the development and function of the shoot apical meristem of microspore-derived embryos of *Brassica napus* ' Topas'. Botany **84:** 371-383.

Rashotte, A.M., Mason, M.G., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2006). A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc. Natl. Acad. Sci. USA **103:** 11081-11085.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J.A. (1999). Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science **283:** 1541-1544.

Sarkar, A.K., Luijten, M., Miyashima, S., Lenhard, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R., and Laux, T. (2007). Conserved factors regulate signalling in *Arabidopsis thaliana* shoot and root stem cell organizers. Nature **446:** 811-814.

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E.H., Schmid, M., Jürgens, G., and Weijers, D. (2010). MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature **464:** 913-916.

Seguí-Simarro, J.M., and Nuez, F. (2008). How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol. Plant. **134:** 1-12.

Soriano, M., Li, H., and Boutilier, K. (2013). Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reprod. **26:** 181-196.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature **403:** 41-45.

Szemenyei, H., Hannon, M., and Long, J.A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. Science **319:** 1384-1386.

Tai, H.H., Tai, G.C., and Beardmore, T. (2005). Dynamic histone acetylation of late embryonic genes during seed germination. Plant Mol. Biol. **59:** 909-925.

Tajima, Y., Imamura, A., Kiba, T., Amano, Y., Yamashino, T., and Mizuno, T. (2004). Comparative studies on the type-B response regulators revealing their distinctive properties in the His-to-Asp phosphorelay signal transduction of *Arabidopsis thaliana*. Plant Cell Physiol. **45:** 28-39.

Tanaka, H., Watanabe, M., Watanabe, D., Tanaka, T., Machida, C., and Machida, Y. (2002). *ACR4*, a putative receptor kinase gene of *Arabidopsis thaliana*, that is expressed in the outer cell layers of embryos and plants, is involved in proper embryogenesis. Plant Cell Physiol. **43:** 419-428.

Tanaka, M., Kikuchi, A., and Kamada, H. (2008). The Arabidopsis histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. Plant Physiol. **146:** 149-161.

Thimm, O., Blasing, O., Gibon, Y., Nagel, A., Meyer, S., Kruger, P., Selbig, J., Muller, L.A., Rhee, S.Y., and Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics

data sets onto diagrams of metabolic pathways and other biological processes. Plant J. **37:** 914-939.

Tian, L., and Chen, Z.J. (2001). Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. Proc. Natl. Acad. Sci. USA **98:** 200-205.

Tsuwamoto, R., Fukuoka, H., and Takahata, Y. (2008). *GASSHO1* and *GASSHO2* encoding a putative leucine‐rich repeat transmembrane‐type receptor kinase are essential for the normal development of the epidermal surface in Arabidopsis embryos. Plant J. **54:** 30-42.

Tsuwamoto, R., Yokoi, S., and Takahata, Y. (2010). *Arabidopsis EMBRYOMAKER* encoding an AP2 domain transcription factor plays a key role in developmental change from vegetative to embryonic phase. Plant Mol. Biol. **73:** 481-492.

Twell, D. (2011). Male gametogenesis and germline specification in flowering plants. Sex. Plant Reprod. **24:** 149-160.

Uddenberg, D., Valladares, S., Abrahamsson, M., Sundström, J., Sundås-Larsson, A., and von Arnold, S. (2011). Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor. Planta **234:** 527-539.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9:** 1963-1971.

Wang, Z., Zang, C., Cui, K., Schones, D.E., Barski, A., Peng, W., and Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell **138:** 1019-1031.

Waterborg, J.H. (1998). Dynamics of histone acetylation in *Chlamydomonas reinhardtii*. J. Biol. Chem. **273:** 27602-27609.

Weijers, D., Schlereth, A., Ehrismann, J.S., Schwank, G., Kientz, M., and Jürgens, G. (2006). Auxin triggers transient local signaling for cell specification in *Arabidopsis* embryogenesis. Dev. Cell **10:** 265-270.

Wu, K., Tian, L., Malik, K., Brown, D., and Miki, B. (2000). Functional analysis of HD2 histone deacetylase homologues in *Arabidopsis thaliana*. Plant J. **22:** 19-27.

Wu, K., Zhang, L., Zhou, C., Yu, C.-W., and Chaikam, V. (2008). HDA6 is required for jasmonate response, senescence and flowering in *Arabidopsis*. J. Exp. Bot. **59:** 225-234.

Xu, C.-R., Liu, C., Wang, Y.-L., Li, L.-C., Chen, W.-Q., Xu, Z.-H., and Bai, S.-N. (2005). Histone acetylation affects expression of cellular patterning genes in the *Arabidopsis* root epidermis. Proc. Natl. Acad. Sci. USA **102:** 14469-14474.

Xu, K., Liu, J., Fan, M., Xin, W., Hu, Y., and Xu, C. (2012). A genome-wide transcriptome profiling reveals the early molecular events during callus initiation in *Arabidopsis* multiple organs. Genomics **100:** 116-124.

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). *HISTONE DEACETYLASE19* is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. Plant Cell **17:** 1196-1204.

Chapter 5

Plasticity in cell division patterns and auxin transport-dependency during in vitro embryogenesis

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Abstract

Cell division during *Arabidopsis* and *Brassica* zygotic embryogenesis is highly regular, but it is unclear whether these conserved divisions are essential for embryo cell fate and functionality. We addressed this question using the *Brassica* microspore embryogenesis system, where the male gametophyte is reprogrammed *in vitro* to form haploid embryos in the absence of exogenous growth regulators. Microspore embryos are formed via two pathways: a zygotic-like pathway, characterized by initial suspensor formation followed by embryo proper formation from the distal cell of the suspensor, and a pathway characterized by initially unorganized embryos lacking a suspensor. Using embryo fate and auxin markers we show that the zygotic-like pathway requires polar auxin transport for embryo proper specification from the suspensor, while the suspensorless pathway is polar auxin transport-independent and marked by an initial auxin maximum, suggesting early embryo proper establishment in the absence of a basal suspensor. Polarity establishment in this suspensorless pathway was triggered and guided by rupture of the pollen exine. Irregular division patterns did not affect cell fate establishment in either pathway. The cell fate and patterning processes that accompany *Brassica* microspore embryogenesis are therefore considerably more flexible than suggested by observations on Arabidopsis zygotic embryogenesis, and imply that additional cell fate and patterning mechanisms operate during plant embryogenesis.

Introduction

The basic body plan of a plant is established during embryogenesis by sequential divisions, which in most angiosperms start with the asymmetric division of the zygote. In the model plant Arabidopsis (*Arabidopsis thaliana*), the subsequent division planes of the embryo are highly regular, making it possible to follow the establishment of the different cell lineages from individual cells. The asymmetric first division of the zygote generates two cells with very different fates. The small apical cell gives rise to the embryo proper, while the larger basal cell divides transversally to produce the suspensor, a mostly extra-embryonic structure, that positions the embryo inside of the seed, provides nutrients and hormones to the embryo proper (Cionini et al., 1976; Nagl, 1990), and contributes to the root meristem (Berleth and Jurgens, 1993; Scheres et al., 1994). Embryos that lack a well-formed suspensor

also show aberrant morphologies in the embryo proper (Lukowitz et al., 2004; Bayer et al., 2009; Ueda et al., 2011), while aberrant development of the embryo proper can trigger ectopic division and embryo formation in suspensor cells (Schwartz et al., 1994; Vernon and Meinke, 1994; Yadegari et al., 1994; Zhang and Somerville, 1997). These observations indicate that there is crosstalk between these two structures, in which the embryo proper inhibits embryo formation in suspensor cells and the suspensor supports growth and polarity establishment of the embryo proper.

Mutant analysis in Arabidopsis suggests that the asymmetric division that generates the initial apical-basal pattern of the embryo is important for subsequent cell fate establishment and morphogenesis, as mutants that fail to establish the correct division plane can show subsequent defects in embryo organization or even developmental arrest (Mayer et al., 1993; Breuninger et al., 2008; Ueda et al., 2011). Likewise, loss-of-function mutants of key suspensor genes in the YODA/GROUNDED pathway disrupt the elongation and first division of the zygote. These mutant embryos lack a well-defined suspensor and both the suspensor and the embryo proper show irregular cell divisions (Lukowitz et al., 2004; Bayer et al., 2009; Jeong et al., 2011).

The hormone auxin is a central regulator of cell division, differentiation and growth, and plays an important role in zygotic embryo patterning, as the majority of embryo pattern mutants studied in Arabidopsis are defective in auxin-related pathways (Wendrich and Weijers, 2013). Auxin accumulation is regulated in part by the PIN family of auxin efflux carrier proteins (Křeček et al., 2009). Differential expression of PIN proteins combined with their polar localisation on the cell membrane generates auxin gradients that specify cell- and organ fates in a context-dependent manner. During embryo development, the PIN7-directed flow of auxin from the basal- to the apical cell of the two-celled embryo establishes the embryo proper, while reversal of this flow at the globular stage by the polar localization of PIN1 and PIN4 towards the hypophysis establishes the basal embryo domain (Friml et al., 2003).

Polar auxin transport plays a major role in the establishment of the apical and basal embryo domains and bilateral symmetry in dicot embryos (Liu et al., 1993; Friml et al., 2003). Auxin transport also provides robustness to the embryo pattern, as it buffers auxin distribution in the embryo to local changes in auxin homeostasis (Weijers et al., 2005). Due to functional redundancy among PIN family members single *pin* mutants only show weakly

penetrant embryo patterning phenotypes (Friml et al., 2003), however, quadruple *pin* mutants (*pin2pin3pin4pin7* and *pin1pin3pin4pin7*) show strong and highly penetrant apical-basal patterning defects that are associated with mislocalization of apical and basal stem cell regulators (Blilou et al., 2005). Global changes in PIN polarity, as in the *gnom* or the *RPS5A::PID* mutant backgrounds, also lead to strong-apical basal patterning defects (Steinmann et al., 1999; Friml et al., 2004).

Many of the Arabidopsis embryo mutants that initially show patterning defects, often recover and are able to develop into functional seedlings. This, combined with the large amount of variation in the organization of the embryo proper and suspensor in species other than Arabidopsis (Rutishauser, 1969; Johri et al., 1992; Yeung and Meinke, 1993; Kaplan and Cooke, 1997; Madrid and Friedman, 2009; Guillon et al., 2012), as well as the observation that many embryos produced in vitro do not show early morphological signs of patterning (Mordhorst et al., 1997; Raghavan, 2004; Bassuner et al., 2007), indicates that ordered cell divisions are not required for embryo patterning and functionality.

Here we used the *in vitro Brassica napus* microspore embryogenesis system to study how non-regular patterns of cell division influence the formation of a functional embryo. In this system embryogenesis can be induced from isolated microspores and bicellular pollen grains by a short heat stress treatment (Custers et al., 1994). *Brassica* zygotic embryos, like those of Arabidopsis, undergo highly regular cell divisions (Tykarska, 1976, 1979). In contrast, the first division during *Brassica* microspore embryogenesis is usually symmetric, and embryo development does not proceed via a regular pattern of cell division. Suspensor development is also not a prerequisite for *Brassica* microspore-derived embryo formation, although suspensor-like structures with varying degrees of organization are observed and can be induced at a high frequency in some genotypes (Hause et al., 1994; Yeung et al., 1996; Ilić-Grubor et al., 1998; Supena et al., 2008).

In this study we show that the two different pathways of haploid *in vitro* embryo development, with and without initial suspensor formation, are marked by differences in auxin response and transport. Both pathways are highly flexible in that they are not compromised by irregular cell divisions. Our data provide insight into how embryo cell fate and patterning are established in the absence of highly regular cell divisions.

Results

Haploid embryo development in the absence of a suspensor

In *B. napus*, male gametophyte development starts with the single-celled microspore, and after two mitotic divisions (pollen mitosis I (PMI) and PMII) results in the formation of a trinucleate pollen grain (Figure 1A). Heat stress induces a sporophytic program in cultured microspores and binucleate pollen that leads to the formation of haploid embryos. Many correlative cell biology observations have suggested that the first characteristic of sporophytic development in microspore culture is the symmetric division of the uninucleate microspore or vegetative cell of the pollen grain, which can be distinguished from the first asymmetric division that characterizes pollen development (Figure 1B) (Fan et al., 1988; Zaki and Dickinson, 1990). In embryos that develop without a suspensor, this initial division is followed by a series of randomly-oriented divisions in which the different cell layers, normally present in zygotic embryos, cannot be recognized (Figure 1C). Further random division of these structures stretches the surrounding exine (pollen coat) until one of the exine locules breaks, releasing a globular-shaped structure (Figure 1D). The embryonic epidermis, the protoderm, forms at this time (Figure 1E), and is followed by histodifferentiation of the major tissue types and organs of the embryo (Figure 1F).

Establishment of embryo identity does not require microspore division

The irregular cell divisions observed during microspore embryogenesis makes it difficult to determine morphologically when a structure first becomes embryogenic. To more precisely define when cultured microspores become committed to the embryo development pathway, we generated *B. napus* lines carrying the *proGRP:GFP-GUS* reporter. This reporter is expressed in *B. napus* at the zygote stage (Li et al., 2014), in both the apical and basal cells of the embryo (Supplemental Figure 1A-C). Its expression gradually becomes restricted to the suspensor and basal cells of the embryo proper and finally to the cells that form the columella root cap of the embryo proper. The *GRP* reporter is not expressed during *in planta* pollen development (Supplemental Figure 1D-H). The percentage of *GRP*-positive structures at day 2 of culture was highly correlated with the number of embryos formed (Supplemental Figure 1I). *GRP* expression is therefore a suitable marker for following the establishment of embryo cell fate in microspore cultures.

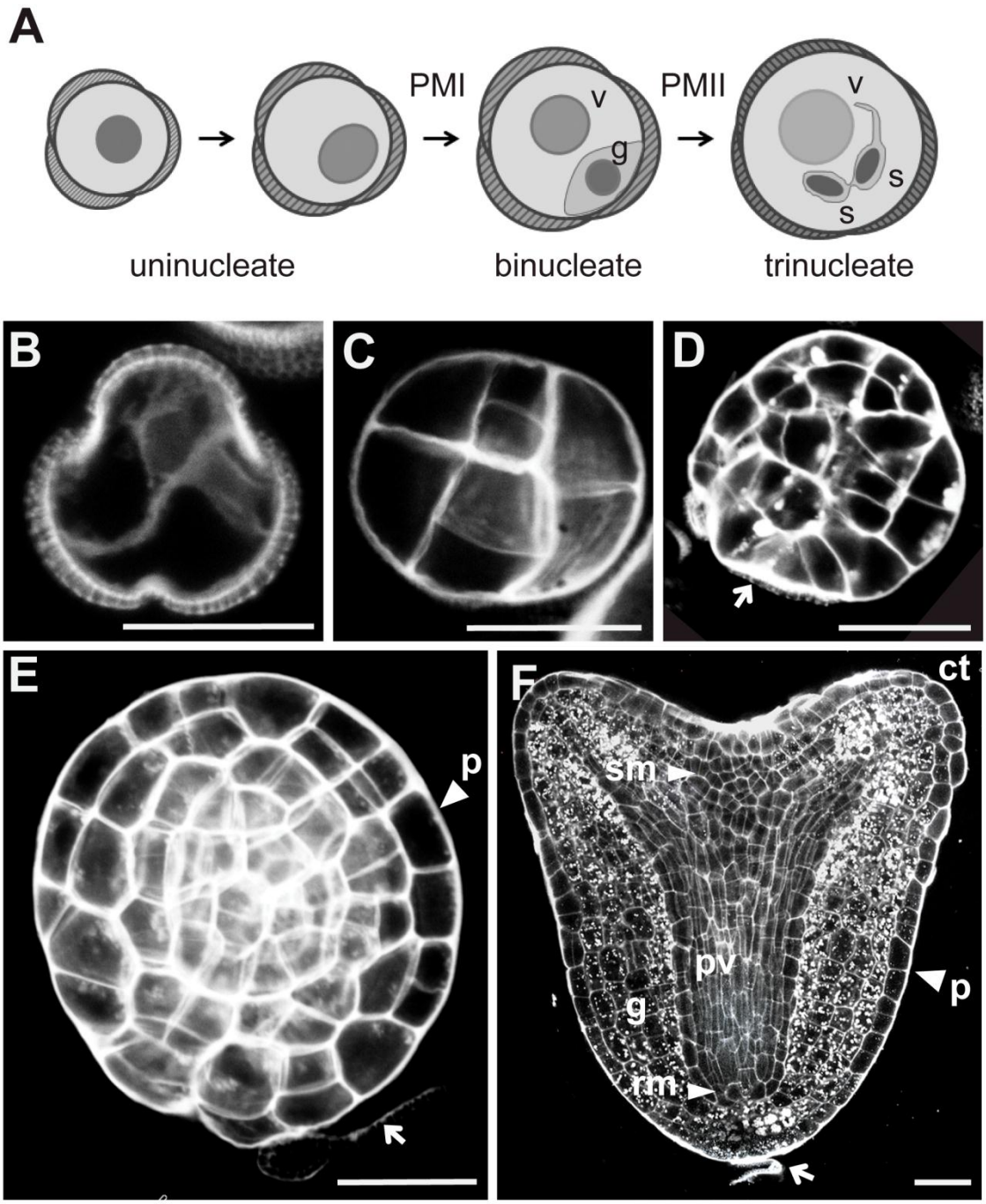

Figure 1. Developmental pathways in microspore culture.

(A) Pollen development. **(B-F)** Histodifferentiated embryo production. **(B)** Symmetrically-divided microspore. **(C)** Pro-embryo enclosed in the exine. **(D)** Globular embryo released from the exine. **(E)** Globular embryo with a well-formed protoderm layer. **(F)** Heart stage embryo. Arrow, exine remnants; c, cell wall; ct, cotyledon; g, ground tissue; p, protoderm; pv, provascular tissue, rm, root meristem; sm, shoot meristem; s, spermatid; v, vegetative cell. Bar=20μm.

We examined *GRP* expression in combination with DAPI staining to define the developmental stage at which the embryogenic program is established in microspore culture. *GRP* reporter expression could be observed as early as one day after the start of culture, in approximately 0.2% of the microspores, and this percentage rapidly increased by the third day of culture to up to 4% of the total population.

Symmetrically divided microspores and the symmetrically-divided vegetative cell of binucleate pollen are reported to form haploid embryos in *B. napus* (Fan et al., 1988; Zaki and Dickinson, 1990). We observed *GRP*-driven GFP expression in these symmetrically-divided cell types (Figure 2A, B), which represented almost 60% of the *GRP*-positive structures (Figure 2G). Notably, *GRP* expression was absent in approximately 40% of the symmetrically-divided cells (Supplemental Figure 1J-K) (Li et al., 2014), suggesting that symmetric division is not an absolute marker for the change in cell fate from pollen to embryo development.

We also observed strong GFP expression in three other types of structures that were not considered in the literature to be embryogenic. These comprised microspores (Figure 2C, G) and pollen vegetative cells (Figure 2D), asymmetrically-divided structures with large and small GFP-positive cells (Figure 2E, F, G), and loosely connected cells that had burst prematurely out of the exine (Figure 2H). In asymmetrically-divided structures the nucleus of the smaller GFP-positive cell resembled a pollen generative cell, but did not show the typical lens shape and DNA compaction found in the generative nuclei of (GFP-negative) pollen (compare Figure 2D with Figure 2E and F). The loosely-connected GFP-positive cells were described previously as ‘non embryogenic’ based on their lack of histodifferentiation (Fan et al., 1988; Telmer et al., 1995; Ilić-Grubor et al., 1998). These clusters increased in cell number during the culture period, forming unorganized masses of round, expanded cells, but eventually died.

Our data suggest that many microspores are initially programmed to develop as embryos, and that this initial switch in developmental pathways can occur in the absence of cell division and independent of the initial division symmetry. However, only ca. 0.5% of the sporophytic structures will eventually form the compact structures that form histodifferentiated embryos (Figure 2I, Li et al., 2014), suggesting that additional signalling events are required to ensure further embryo growth and differentiation.

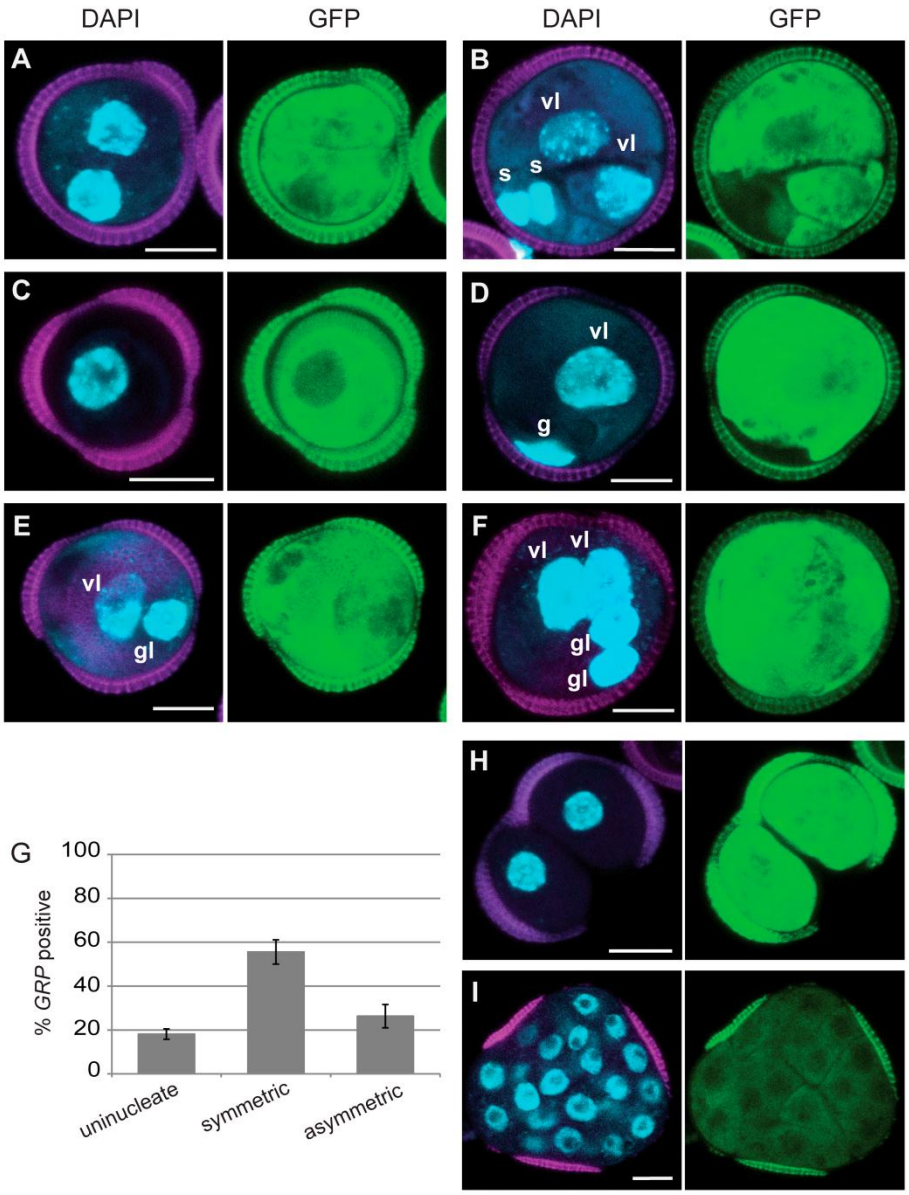

Figure 2. Embryo identity in microspore culture is not dependent on cell division or division symmetry.

(A-F) *proGRP:GUS-GFP* embryo marker expression after three days of culture. DAPI staining (blue), autofluorescence (magenta) and GFP expression (green). **(A)** Symmetrically-divided microspore showing *GRP* expression in both cells. **(B)** *GRP* expression in the two symmetrically-divided vegetative-like cells, but not the smaller generative/sperm-like cells. **(C)** *GRP* -positive microspore. **(D)** *GRP* expression in the vegetative-like cell, but not in the smaller generative-like cell. **(E-F)** *GRP* expression in both the vegetative- and generative-like cells. **(E)** Asymmetrically-divided bicellular structure. **(F)** Multinucleate structures. Note that the generative-like nuclei in (E) and (F) are larger and the DNA is less condensed than in (D). **(G)** Proportion of microspores, and symmetrically- and asymmetrically divided structures that express *proGRP:GFP-GUS* at day 3 of culture. Structures where *GRP* was only expressed in the vegetative cell of binucleate (D) were not included in the graph. **(H-I)** *GRP*-positive structures found at 6 days of culture. **(H)** Sporophytic structure prematurely released from the exine. **(I)** Proembryo enclosed in the exine. Error bars in (G) indicate the standard error of three replicates. vl, vegetative-like nucleus; gl, generative-like nucleus. Bar=10μm.

Auxin signalling marks embryogenic microspores

In view of the important role of auxin in zygotic embryo development, we examined the timing and spatial distribution of the auxin response reporter *proDR5:GFP* and the Arabidopsis *proPIN1:PIN1-GFP* and *proPIN7:PIN7-GFP* reporters to gain insight into auxin dynamics during the initiation of *B. napus* microspore embryo development. The expression patterns of these reporters are conserved between Arabidopsis and *B. napus* zygotic embryos (Supplemental Figure 2A-C), except that in *B. napus proDR5:GFP* expression was only observed in the embryo proper from the octant stage of development onward, whereas in Arabidopsis *DR5* expression is first detected in the embryo proper at the one-cell stage (Friml et al., 2003). In *B. napus*, neither the PIN1-GFP protein nor the PIN7-GFP protein was observed during male gametophyte development *in planta*. *proDR5*:*GFP* expression was observed during male gametophyte development *in planta*, but at an earlier stage than is used for microspore culture (Supplemental Figure 2D). In conclusion, none of the auxin reporters were expressed in any of the gametophytic structures that are present in microspore cultures.

During the first three to five days of microspore culture, neither PIN1-GFP nor PIN7-GFP was observed in gametophytic structures or randomly divided, suspensorless sporophytic structures. These data suggest that suspensorless proembryos growing inside the exine do not show the same early apical-basal specification that occurs in zygotic embryos.

Weak *DR5* expression was first observed around the third day of culture in up to 0.2% of the microspore population. These GFP-positive structures were either uninucleate or had divided symmetrically (Figure 3A, B). Notably, *DR5* expression was very weak and could not be detected in compact embryo structures after the two-celled stage (Figure 3C). The proportion of *proDR5:GFP*-expressing cells was always lower than the ca. 4% marked by *GRP*, but in line with the final embryo yield (Supplemental Figure 1I). Unlike *GRP* expression, *DR5* expression did not mark the asymmetric pollen-like structures, nor did it mark the loosely connected cell clusters that released prematurely from the exine (Figure 3D). These results show that a transient auxin response marks a subset of embryogenic cells. In analogy with the *DR5* maximum in the embryo proper of zygotic embryos (Friml 2003), *DR5* expression in embryogenic microspores suggests that these cells are initially programmed as an embryo proper. The failure of certain *GRP*-marked embryogenic structures (i.e. asymmetrically divided microspores and callus-like cells) to establish an auxin response suggests that these

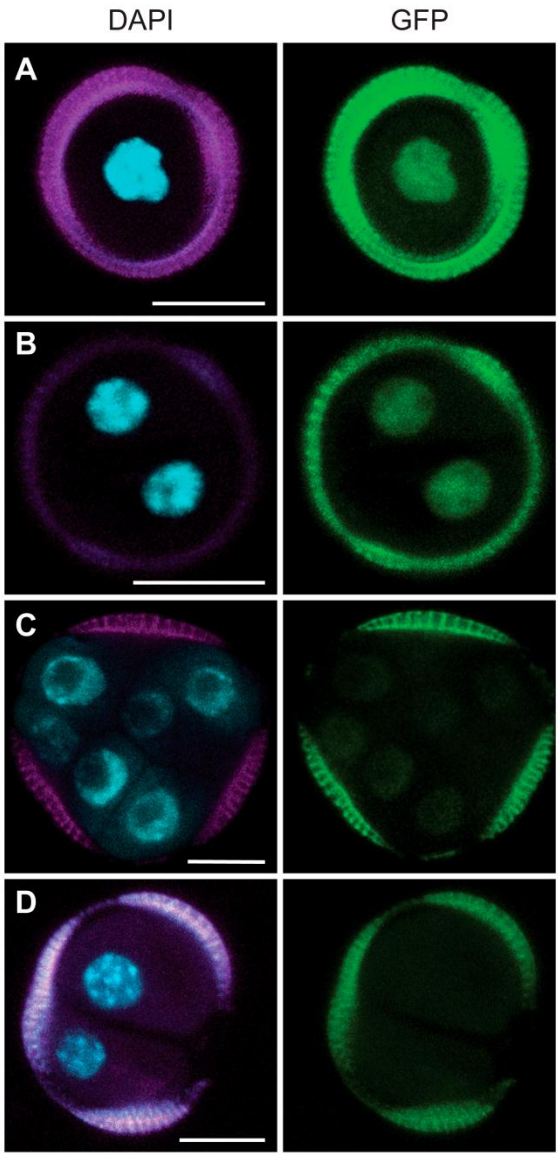

Figure 3. *proDR5:GFP* expression marks embryogenic cells.
(A) Microspore-like structure**. (B)** Symmetrically-divided microspore. **(C)** *DR5* expression in compact multinucleate structures. **(D)** *DR5* is not expressed in multinucleate structures that emerged prematurely from the exine DAPI staining (blue), autofluorescence (magenta) and GFP fluorescence (green). Bar=10μm**.**

structures are associated with a different pathway of haploid embryo development in which the formation of an embryo proper is not initiated or is delayed.

Embryo polarization follows exine rupture

Early growth in suspensorless embryos causes stretching of the exine, which eventually breaks. After exine rupture, the apical and basal poles of the haploid embryo become morphologically apparent through the formation of cotyledons and elongation of the embryo axis (Yeung et al., 1996). The position of the remaining exine pieces highly correlated with the basal region of the embryo (Figure 1E) (Ilić-Grubor et al., 1998; Supena et al., 2008; Tang et al., 2013), and is therefore a much earlier marker for polarity establishment than apical growth and axis elongation. This polarisation was also marked by the accumulation of

large starch granules at the future basal pole of the embryo. Starch grains were abundant throughout the haploid embryo prior to exine rupture, but upon exine rupture they accumulated predominately in the cells that are located away from the site of exine rupture (i.e. the future basal pole of the embryo) (Figure 4A) (Hause et al., 1994).

Unlike the early stages of suspensorless microspore embryo development, the morphological changes that took place after release of the embryo from the exine were similar to those that take place during zygotic embryogenesis. First, the protoderm was established (Figure 4A), followed by a triangle/transition stage in which the area surrounding the apical pole grew, giving rise to a bilaterally polarized embryo (Figure 4A). The provasculature in triangle/transition stage embryos was free of starch, while the basal pole was marked by the accumulation of large starch grains (Figure 4A). Later, outgrowth of the cotyledons marked the establishment of bilateral symmetry (Figure 1E). The root meristem was not as well defined in microspore embryos as in zygotic embryos, but could be recognized at the early heart stage as a group of isodiametric cells below the provasculature that were devoid of starch, and that were often larger than neighbouring cells (Figure 1E) (Yeung et al., 1996).

We followed the *GRP*, *PIN* and *DR5* reporters in microspore embryos as they emerged from the exine and underwent histodifferentiation. *GRP* was initially expressed throughout the embryo clusters while they were still enclosed by the exine (Figure 4B). *GRP* expression at this stage was much weaker than in few-celled embryos. After exine rupture, *GRP* expression disappeared from the cells at the rupture site and from the inner cells of the embryo, remaining localized at the site of the presumptive basal pole (Figure 4B). Later at the globular and transition stages, *GRP* expression was very weak and marked the basal cell tiers of the embryo and the future columella (Figure 4B). At this stage, *GRP* expression was highly correlated with the presence of pollen wall remnants on the surface of the embryo (Supplemental Figure 3A).

During zygotic embryo development, *PIN1* is expressed in the embryo proper from the eight-cell stage embryo onward (Friml et al., 2003). PIN1-GFP was not observed prior to exine rupture in microspore embryos lacking suspensors. Rather, PIN1-GFP was first observed in cells that protruded through the pollen pores at the time that the exine started to break (Figure 4C). In embryos that had completely burst free of the exine, PIN1-GFP expression was strong on the outer cell layer in the apical region and in the inner cells, with

no clear cellular polarization, and gradually marked the formation of provascular strands at the late globular/transition stage (Figure 4C). At the heart stage, *proPIN1:PIN1-GFP* was expressed in the same pattern in microspore and zygotic embryos, in the epidermal cell layer, where the fusion protein showed clear apical polarization, and in the provascular tissue, where the fusion protein showed a rather basally-oriented polarity (Supplemental Figure 2A and 3B). In contrast to *PIN1*, *proPIN7:PIN7-GFP* expression was not observed in suspensorless embryos.

Together, these data suggest that in suspensorless embryos, *PIN1* and *GRP* expression mark the formation of the apical and basal domains of the embryo, respectively, that apical-basal axis determination takes place late in embryo development, after exine rupture, and that *PIN7* expression does not mark the basal cell lineage.

These data were supported by time-lapse imaging of the *proDR5:GFP* reporter during exine rupture (Figure 4D). As noted above, *DR5* expression was very weak or could not be detected in compact embryo structures after the two-celled stage, but became strongly expressed in the inner cells of embryos comprising 15-17 cells that showed stretching of the exine (Figure 4D, 0 h). The site of *DR5* expression did not change when the exine broke, but rather became weaker (Figure 4D, 24 h), then disappeared from the site where the pollen wall broke (Figure 4D, 48 h), and eventually became stronger again and restricted to a few cells that mark the site of presumptive root meristem (Figure 4D, 72 h). These results show that in microspore embryos that lack a suspensor, a basal auxin maximum is established after exine rupture, in the same spatial pattern as the *GRP* reporter. However, polarization of DR5 expression to the basal pole occurs slowly, while *GRP* expression disappears more rapidly from the inner and apical cells.

Exine rupture and apical-basal polarity establishment are independent of polar auxin transport

PIN1-GFP was localized to the sites of pollen wall rupture and later marked the apical pole of the embryo. This observation, together with the basal auxin maximum established after exine rupture, suggests that PIN1 directs auxin away from the apical region of the embryo. However, it is not clear whether (PIN-directed) polar auxin transport (PAT) is the driving force behind exine rupture. We used the PAT inhibitor, N-1-naphthylphthalamidic acid (NPA)

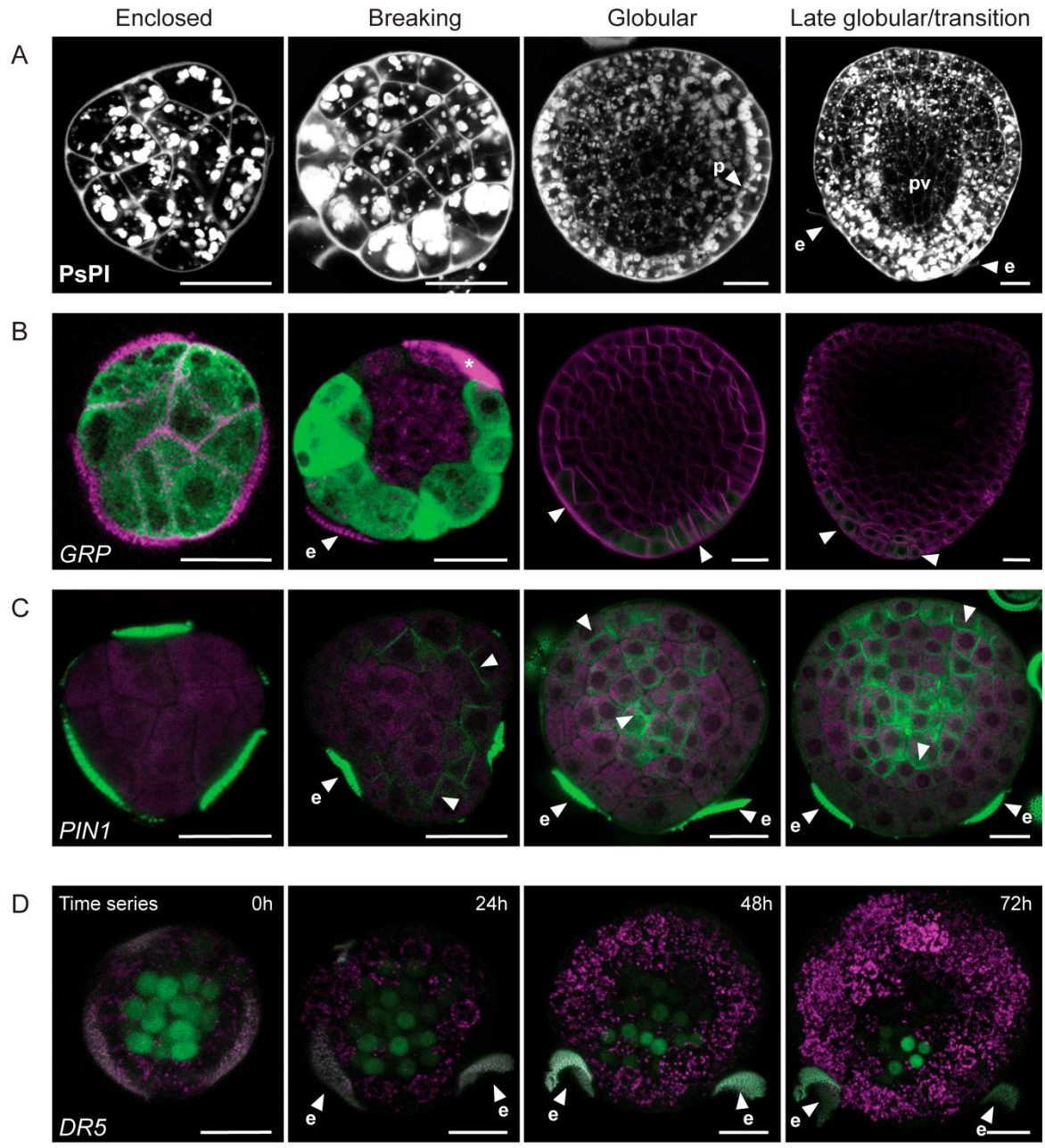

Figure 4. Embryo polarization occurs after exine rupture.

(A-C) Four successive stages of microspore embryo development are shown from left to right: embryos still enclosed in the exine; embryos shortly after the exine has broken at one of the locules; globular stage embryos released from the exine; and late globular to transition stage embryos. **(A)** Starch grain accumulation (white globules) and cell walls (white) visualized by Ps-PI staining. **(B)** *proGRP:GUS-GFP* expression. Membrane staining by FM4-64 (magenta) and GFP fluorescence (green). **(C)** *proPIN1:PIN1-GFP* expression. PI counterstain (magenta) and GFP fluorescence (green). **(D)** Time-lapse image of *proDR5:GFP* expression at the time of exine rupture. Exine and chloroplast autofluorescence (magenta) and GFP fluorescence (green). Arrow heads, GFP localization; e, exine; p, protoderm; pv, provascular tissue. The asterisk marks cellular debris stained by FM4-64. Bar=20μm.

to understand the relationship between PAT and exine rupture in suspensorless microspore embryo development.

Continuous treatment of cultured DH12075 microspores with NPA consistently reduced the proportion of cells that developed into embryos, although this trend was only statistically significant in 20 µM NPA treatments (Figure 5A). Application of NPA to microspore cultures did not change the proportion of *GRP*-positive microspores at day 3 of culture (Figure 5B), suggesting that the effect of NPA on embryo yield occurs later in development. As previously reported, NPA induced the formation of morphologically defective cup-shaped embryos or embryos with fused cotyledons (Figure 5C, D) (Hadfi et al., 1998; Friml et al., 2003; Hakman et al., 2009). Despite these defects, almost all NPA-treated embryos had clear root and shoot poles (Figure 5C, D), but the function of the meristems was compromised: less than 10% of the embryos that developed in the presence of NPA produced roots after 5 days on regeneration medium, while roots were produced in more than 80% of the control embryos (Figure 5E, F). After 25 days on regeneration medium, some NPA-treated embryos had formed roots indirectly, either from the basal part of the hypocotyl, or from callus tissue in the basal region of the embryo. None of the embryos from NPA-treated cultures produced a shoot from the apical meristem, compared to 60% in the control cultures (Figure 5E, F).

Exine rupture in NPA-treated cultures was similar to that in control cultures. *GRP* expression and starch accumulation were also properly localized to the basal pole after exine rupture in NPA-treated embryos (Figure 6A-F). However, starch also accumulated in the inner cells at the basal side of the provascular tissue, adjacent to the root pole, suggesting differentiation of vascular stem cells (Figure 6F). In contrast to GRP expression, *DR5* expression was perturbed in embryo cultures treated with NPA. At the time of exine rupture, *DR5* was expressed throughout the embryo rather than being localized to the inner cells of embryos, as in the control cultures (Figure 6G, J). After exine rupture, *DR5* expression was extremely weak and randomly-localized throughout the embryo, rather than strongly expressed at the basal pole, as in control embryos (Figure 6H, K). In NPA-treated tube-shaped embryos, *DR5* expression was localized to a broad region of the basal area and then eventually disappeared (Figure 6I, L). This data, together with our morphological observations, suggests that PAT does not direct exine rupture, nor does it play a role in the initial establishment of apical-basal polarity in suspensor-less embryos. Rather, PAT appears

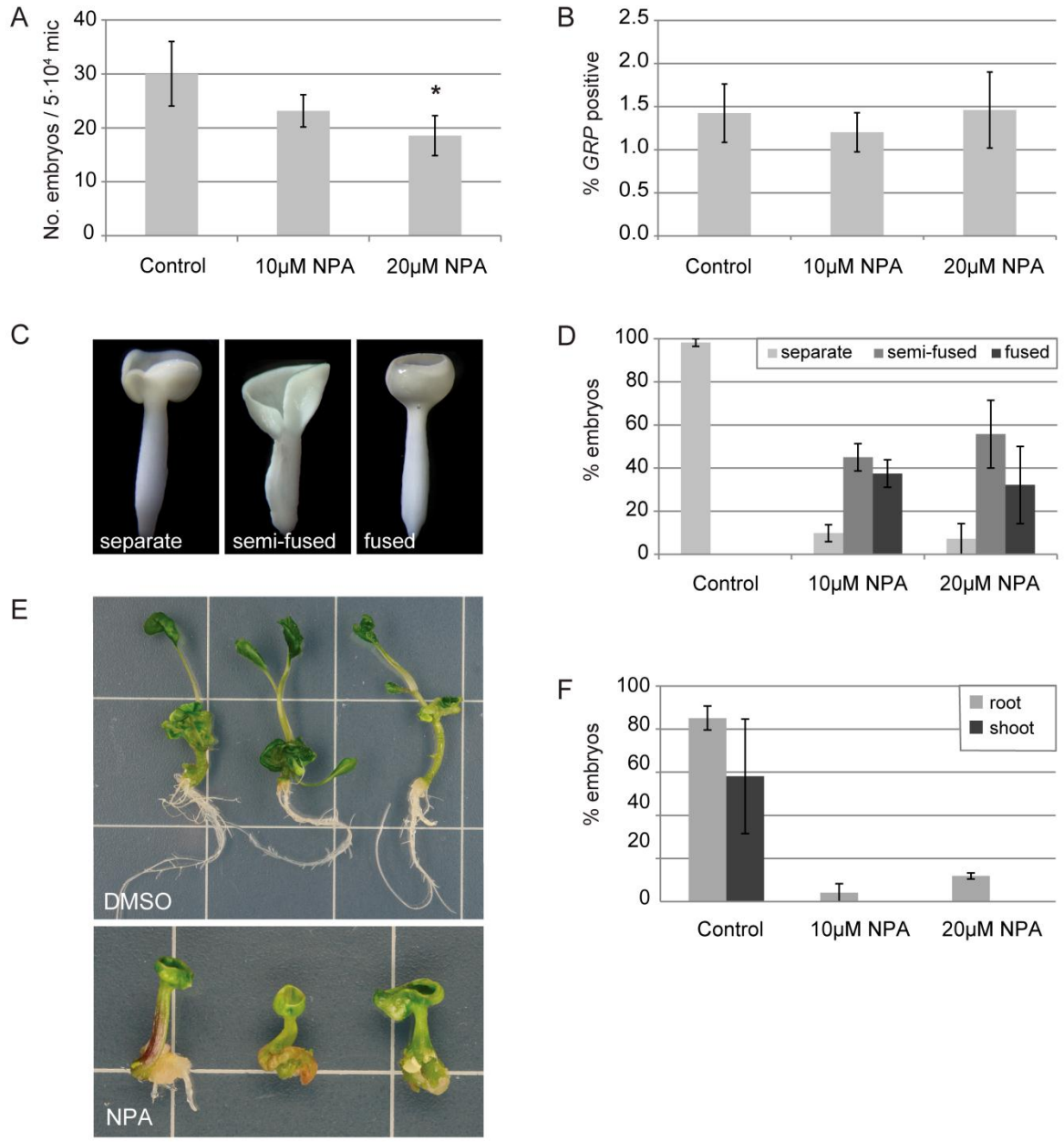

Figure 5. Inhibition of polar auxin transport induces defects in embryo development, but not embryo initiation. **(A)** Effect of continuous treatment with NPA on embryo yield. Cultures were scored at day 25. **(B)** Percentage of *proGRP:GUS-GFP* expressing microspores at day 3 of culture. **(C)** Cotyledon morphologies: left, embryos with two, separated cotyledons; centre, embryos with semi-fused cotyledons in which only one cotyledon boundary is observed and; right, embryos with completely fused or collar-shaped cotyledons. **(D)** Relative abundance of the different embryo morphologies shown in (C) after treatment with NPA. **(E)** Control embryos gave rise to a high frequency of plantlets containing both a root and a shoot (upper panel), while NPA-treated embryos showed reduced root growth and failed to form shoots (lower panel). **(F)** The relative frequency of root (light grey bars) and shoot (dark grey bars) formation in control and NPA-treated embryos in a germination assay. mic: microspores. Error bars indicate the standard error three to 11 replicates. *, statistically significant difference with control $p<0.05$ (ANOVA).

to be required to focus the initial auxin maximum at the basal pole, and this seems to be important for the transition from radial to bilateral symmetry, and for the establishment of well-defined poles with functional shoot and root meristems.

Suspensor-derived embryogenesis is PAT-dependent

The efficiency of suspensor-bearing embryo production in microspore culture is genotype-dependent. Suspensor-bearing embryos are efficiently induced in the model *B. napus* line Topas DH4079 (Joosen et al., 2007; Supena et al., 2008) by exposing the microspores to a shorter heat-stress treatment. In this genotype, a long uniseriate cell filament emerges through a pore in the exine wall. The embryo proper is initiated by a transverse division of the suspensor cell that is most distal to the exine remnants, and divides in a more ordered pattern, similar to the zygotic embryo. A wide range of abnormal suspensor morphologies are also observed, ranging from multiple files of cells to small protrusions (Supena et al., 2008). Unlike the model line Topas DH4079, suspensor-bearing embryos are only found in low percentage of the population in the DH12075 line used to generate the reporter lines (ca. 1.5% of the embryos), and were more often short and contained additional cell files or longitudinal divisions (Figure 7A-D). These abnormally-formed suspensors have been associated with a poorly-organized embryo proper (Supena et al., 2008).

We used the embryo and auxin reporter lines to follow the establishment of the apical (*PIN1*) and basal (*PIN7, DR5, GRP*) tiers and histodifferentiation events in suspensor-bearing microspore-derived embryos. As in the zygotic embryo, *GRP* was expressed in all cells of the suspensor (Figure 7E) and was confined to the basal-most tier of the embryo proper from the early globular stage onward (Figure 7F, G). The expression of the *DR5* and *PIN* reporters in suspensor-bearing embryos was similar to their expression in zygotic embryos, with *proPIN7:PIN7-GFP* expressed in the suspensor (Figure 7H-J) and *DR5* and *proPIN1:PIN1-GFP* expressed in the embryo proper (Figure 7K-M). This data provides further support that suspensor-bearing embryos follow a zygotic embryo-like program, even though the embryo proper is derived from the apical cell of a multicellular suspensor, rather than from an asymmetric division of the zygote.

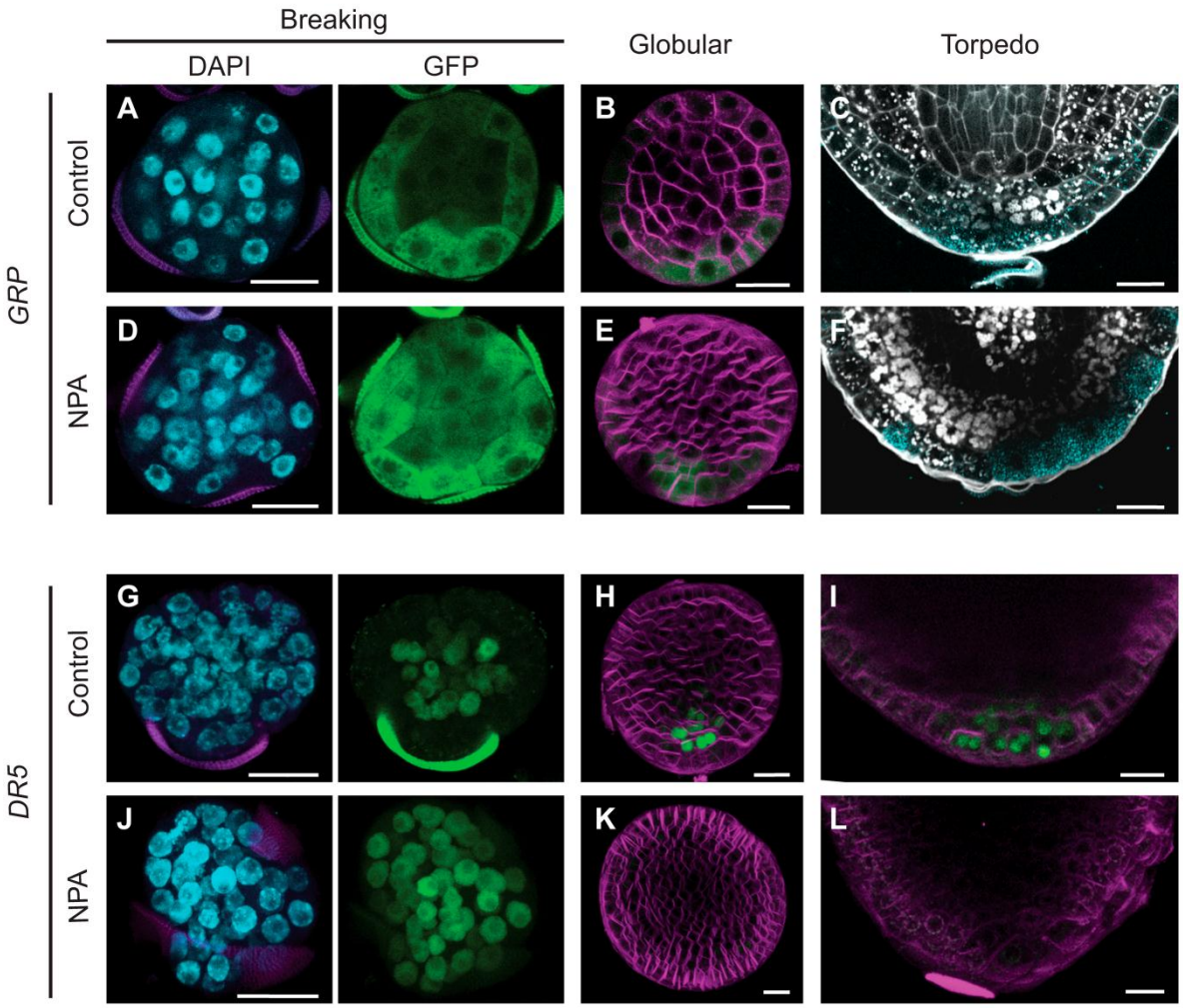

Figure 6. Auxin polar transport is not required for microspore embryo polarization.

(A-F) Establishment of the embryo basal domain, marked by *proGRP:GFP-GUS*. **(G-L)** Localization of auxin response maxima by *proDR5:GFP* expression. Comparison of control and NPA-treated embryos at the stage where the exine starts to break (A, D, G, J), at the globular stage after release from the exine (B, E, H, K), and in the root pole at the torpedo stage at the torpedo stage (C, F, I, L). DAPI staining of the nuclei (blue) and GFP expression (green) are shown separately. FM4-64 (magenta) was used to stain membranes in globular stage embryos. GUS staining of *proGRP:GFP-GUS* lines (light blue) was combined with starch and cell wall localization by PsPI staining (white). Bar=20μm.

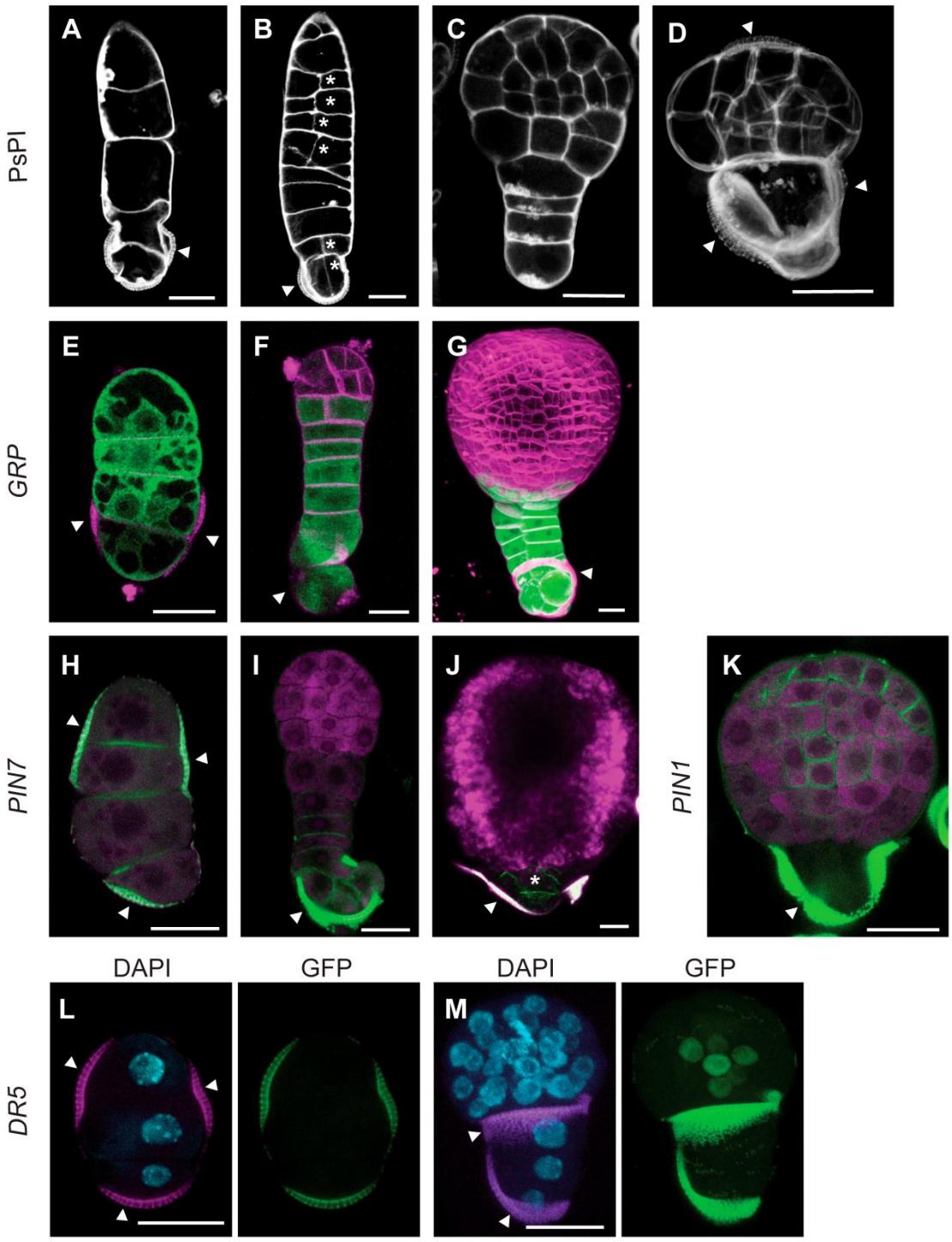

Figure 7. Domain specification in suspensor-bearing microspore embryos is similar to zygotic embryos.

(A-D) Suspensor morphologies observed in microspore culture. **(A)** Uniseriate suspensor. **(B)** Suspensor with aberrant longitudinal divisions (asterisk). **(C)** Microspore embryo with suspensor and embryo proper. **(D)** Microspore embryo with two domains, enclosed in the exine. Arrowheads point to the three exine locules enclosing the proembryo. **(E-G)** *proGRP:GFP-GUS* expression. GFP is shown in green and FM4-64-stained membranes in magenta. **(E)** Uniseriate suspensor. **(F)** Supensor-bearing embryo at the early globular-stage. **(G)** Transition-stage suspensor-bearing embryo, with two cell files. **(H-J)** *proPIN7:PIN7-GFP* expression (green) in

the suspensor. Propidium iodide stain or autofluorescence (magenta). **(H)** Uniseriate suspensor. **(I)** Globular stage embryo with suspensor. **(J)** Transition stage embryo with a rudimentary suspensor. **(K)** *proPIN1:PIN1-GFP* expression (green) in a globular stage suspensor-bearing embryo. **(L-M)** *proDR5:GFP* expression (green) in the embryo proper. DAPI nuclear stain (blue) and autofluorescence (magenta). **(L)** *DR5* is not expressed in the suspensor. **(M)** Suspensor-bearing embryo at the globular stage showing *DR5* expression in the inner cells at the basal region of the embryo proper. Arrow head, exine. Bar=20μm.

We also detected PIN7-GFP in embryos that contained a small suspensor-like protrusion at the basal end of the embryo, rather than a file of cells (Figure 7D, J). These embryos comprised approximately 10% of the embryo population, and were characterized by two heterogeneous domains, one comprising larger vacuolated cells (Figure 7D), in which PIN7-GFP was observed (Figure 7J), and one comprising smaller and more compact cells (Figure 7D) in which PIN1-GFP was observed (Figure 7K). PIN1-GFP and PIN7-GFP could be observed occasionally in a localized region in embryos that were still enclosed in the exine (Supplemental Figure 4). These two domains are reminiscent of the smaller embryo proper cells and the larger suspensor-like cells. *PIN7* expression in the larger and more vacuolated basal cells suggests that these cells function as a suspensor, despite being more rudimentary in structure, and by extension, that unlike zygotic embryo development, suspensor establishment during haploid embryo development does not rely on a highly regular pattern of cell divisions.

The presence of PIN7-GFP in suspensors suggests that PAT plays an important role in this developmental pathway. Addition of NPA to microspore cultures of genotype DH4079, which shows high percentage of suspensor formation, did not have a significant effect on the proportion of initial sporophytic divisions or suspensor development (Figure 8A). However, after 20 days of culture, NPA treatment significantly reduced the final embryo yield in cultures with a large proportion of suspensors (Figure 8B). Interestingly, NPA did not affect the ability of DH4079 microspores to develop suspensors, but rather it specifically affected the ability of the distal cell of the suspensor to form the embryo proper, as shown by the reduction in the number of embryos with a suspensor (Figure 8C). At later stages, a higher proportion of embryos with cup-shaped or fused cotyledons was observed after NPA treatment, as in line DH12075, although the effect of NPA on line DH4079 was more pronounced, with a higher proportion of completely cup-shaped cotyledons, as well as the appearance of pin-shaped embryos (Figure 8D).

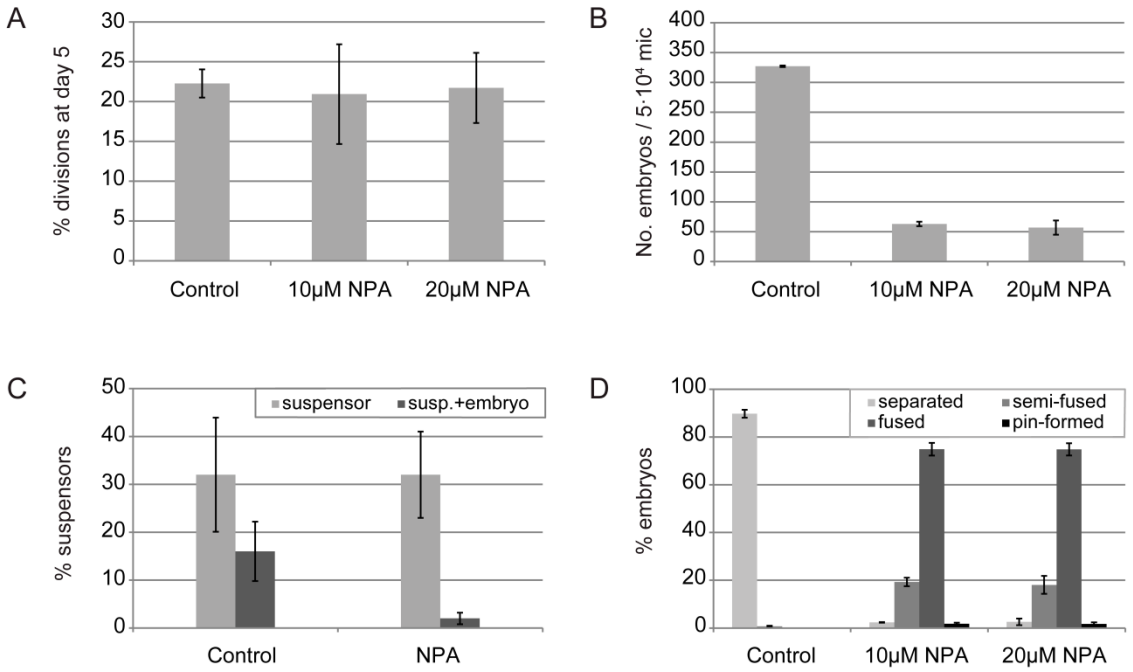

Figure 8. Polar auxin transport is required for embryo proper formation in suspensor-bearing embryos. **(A)** Percentage of sporophytic divisions at day 5 of culture in control and NPA-treated cultures. **(B)** Number of embryos formed in control and NPA-treated cultures at day 25 of culture. **(C)** Percentage of structures with only a suspensor or with both a suspensor and an embryo proper in control and NPA-treated cultures. The data was calculated relative to the total number of sporophytic structures. **(D)** Relative abundance of cotyledon morphologies after treatment with NPA. The morphologies are as in Fig. 5C: embryos with two separated cotyledons, with semi-fused cotyledons in which only one boundary is observed, with completely fused or collar-shaped cotyledons, and embryos lacking cotyledons (pin-formed). mic: microspores. Error bars indicate the standard error of three replicates.

Our data suggest PAT is not required for suspensor formation, but rather, for the establishment of the embryo proper from the suspensor. This observation, combined with our data on development of embryos lacking a suspensor, suggest that differences in auxin response and transport mark the two different pathways of microspore embryo development.

Discussion

Microspore-derived embryos can be obtained via two pathways, one pathway where embryo development occurs in the absence of a suspensor, and a second pathway that starts with the development of a suspensor that eventually forms an embryo proper. Here

we show that in both pathways, the regular pattern of cell division seen in zygotic embryos is not required to establish embryo identity, nor is it required later, to establish the apical and basal domains of the haploid embryo. More importantly, we show that the two pathways are marked by differences in auxin response and transport. Embryogenic microspores that develop without a suspensor are marked by an initial auxin response and do not require PAT for embryo induction, but do require PAT later, for meristem formation and positioning of the cotyledon primordia. In contrast, in the other pathway, suspensor development is not preceded by an auxin response, and PAT is required for embryo proper formation at the distal end of the suspensor. Our observations suggest that plant embryo developmental pathways can be highly flexible and driven by different mechanisms within the same species.

Embryonic cell fate is uncoupled from cell division

During plant and animal development, new cell fates can be established following an initial period of cell proliferation after cells leave the stem cell niche (transit amplifying cells) or through an asymmetric (formative) cell division. Formative divisions that generate two cells with different identities are important to establish the different tissues and organs, not only during plant embryogenesis (epidermis, hypophysis), but also later in development (root cells, lateral roots, stomata, pollen) (De Smet and Beeckman, 2011).

The change in developmental fate from pollen to haploid embryo development was previously thought to be marked by a change in the division plane from an asymmetric pollen-like division to a symmetric division (Fan et al., 1988; Zaki and Dickinson, 1990). We showed, using embryo cell fate markers, that the switch from pollen to embryo identity in *B. napus* microspore culture is uncoupled from cell division, and occurs in both symmetrically- and asymmetrically-divided cells. These observations are in agreement with comparative studies on zygote embryo development in different plant species, which show that the division plane of the zygote is a consequence of its existing polarity and cell shape, suggesting that division planes *per se* do not direct embryo cell fates (Kaplan and Cooke, 1997).

In most species, including *Brassica*, it is the microspore or vegetative cell that contributes to haploid embryo development (Fan et al., 1988); there are very few examples in the literature where embryos originate from the generative cell of cultured pollen (Sunderland and Evans, 1980; Raghavan, 1986). In microspore culture, embryogenic, asymmetrically

divided microspores were characterized by a larger vegetative-like nucleus, and a smaller generative-like nucleus that showed a lower degree of chromatin condensation than in the generative cells of pollen, suggesting that the activation of embryo gene expression in generative-like cells is associated with their change to a more vegetative cell-like fate. Nonetheless, neither symmetric division nor partial decondensation of the chromatin of generative-like nuclei seems to be sufficient for induction of embryo gene expression, since a considerable proportion of this type of divided microspores did not show *GRP* expression. In agreement, symmetrically divided microspores in tobacco and Arabidopsis can sustain pollen development (Eady et al., 1995; Touraev et al., 1995; Park et al., 1998; Park et al., 2004). Thus, the variable division symmetry observed in embryogenic microspores seems to be determined by existing cell polarity cues in the microspore/pollen and is not by itself sufficient to trigger embryo identity.

Auxin response reports embryogenesis competent microspores

An auxin response is first observed at the single celled stage in the suspensorless embryo pathway. This developmental program is different from the Arabidopsis (Friml et al., 2003) or *B. napus* zygotic embryogenesis program, and from the pathway in which haploid embryos develop with suspensors, where an auxin response is only observed in the embryo proper. This suggests that microspore-derived embryos that develop without a suspensor are already programmed as an embryo proper. Auxin is not added to the tissue culture medium, implying that these haploid embryos accumulate auxin, either through de novo biosynthesis or through deconjugation of existing auxin pools (Rosquete et al., 2012; Korasick et al., 2013).

The auxin response marked by *DR5* expression was absent in the asymmetrically-divided cells and the loose, callus-like structures that fail to develop into histodifferentiated embryos, although these structures expressed the *GRP* embryo reporter, as well as *LEAFY COTYLEDON1*, a key regulator of embryo growth and maturation (Li et al., 2014). Thus *DR5* expression appears to mark a subset of embryogenic cells that enter a specific developmental pathway. The lack of auxin response in asymmetrically-divided and callus-like embryogenic structures suggests that, in addition to a switch to embryonic cell fate, an initial auxin response is required to instruct the future differentiation of the embryo.

Polarity establishment following exine rupture

The site of exine rupture plays an important role in polarity establishment in microspore-derived embryos. Cell elongation and apical-basal cell fates in microspore embryos can be induced by early, controlled exine rupture, supporting the role of external positional cues in cell fate establishment (Tang et al., 2013). We have shown that PIN1 first accumulates on the cell membrane at the sites of exine rupture. Recently, it was shown that PIN1 expression and membrane localization can be directed by mechanical signals (Heisler et al., 2010; Nakayama et al., 2012), which are transduced by changes in microtubule orientation and plasma membrane properties. PIN1 membrane localization increases in tomato shoot apex cells when cell turgor and membrane tension are raised (Nakayama et al., 2012). In line with this, during microspore embryogenesis PIN1-GFP is not observed in the tightly contained and growth-constrained multicellular structures enclosed in the exine, but only becomes apparent at the time of rupture, when the external pressure from the exine is released and the embryo cells expand.

The polar secretion and localization of different membrane or cell wall components might also be involved in early embryo polarization. In the brown algae *Fucus*, embryo polarization starts with labile polarization of calcium channels and is stabilized by polarized secretion, producing regions with distinct cell wall properties that are determinant for cell fate specification (Berger et al., 1994; Shaw and Quatrano, 1996). Differential membrane and cell wall properties induced by exine rupture could also give rise to initial positional and polarization cues.

Research on mechanical, cell wall and auxin signalling processes during microspore embryogenesis can shed light on how mechanical stimuli are perceived in plants and their relevance in zygotic embryo patterning. The difference in timing between the establishment of embryonic fate and histodifferentiation in microspore embryos offers the opportunity to characterize these processes independently from each other.

Differential PAT-dependency of the two haploid embryogenesis programs

PAT is a major regulator of apical-basal axis establishment during zygotic embryogenesis. Inhibition of PAT by NPA in Arabidopsis and *Brassica juncea* zygotic embryos causes defects in apical-basal axis establishment, as well as radial patterning and cotyledon formation (Liu et al., 1993; Hadfi et al., 1998; Friml et al., 2003; Weijers et al., 2005). NPA treatment of

cultured *B. napus* microspores indicated that PAT was required to produce functional meristems in suspensorless embryos, but was not required to establish the embryonic apical-basal axis; apical and basal poles were recognizable and basal pole markers (*GRP* and starch accumulation) were not disrupted. By contrast, PINs and PAT are required to specify the shoot and root pole in Arabidopsis zygotic embryos (Friml et al., 2003; Blilou et al., 2005; Robert Boisivon et al., 2013). Our results point to an alternative mechanism of cell fate determination in the embryo that is independent of PAT.

In contrast to suspensorless embryos, suspensor-bearing microspore embryos develop in a similar fashion to zygotic embryos with respect to *PIN* and *DR5* expression. In zygotic embryos, an apical, PIN7-directed flow of auxin is required for the formation of the embryo proper (Friml et al., 2003). NPA inhibition of PAT has not been applied to preglobular stages of zygotic embryos, but when applied at later stages of development (globular or transition) NPA induces severe defects in cotyledon outgrowth and mild defects in shoot and root regeneration (Liu et al., 1993; Hadfi et al., 1998). However, single and higher order *pin* mutants do show early apical and/or basal embryo division defects (Friml et al., 2003; Blilou et al., 2005; Vieten et al., 2005), indicating an important role for PAT in early zygotic embryo patterning. Inhibition of PAT in suspensor-bearing microspore embryos did not interfere with the initiation and specification of suspensor cell fate, but rather inhibited the ability of the distal cell of the suspensor to form the embryo proper. This phenotype is strikingly similar to that of early *pin7* mutant embryos, which frequently lack an embryo proper and/or an apical *DR5* maximum, but eventually become rescued by the onset of *PIN1* and *PIN4* expression (Friml et al., 2003). Our results support previous observations in Arabidopsis zygotic embryos that suggest that the PIN7-directed flow of auxin from the suspensor to the apical cell is important for specification of the embryo proper (Friml et al., 2003; Robert et al., 2013).

Intriguingly, the early *DR5* and *PIN1* expression patterns are maintained in microspore embryos even when the cellular organization of the suspensor or the embryo proper is aberrant. Cell identities are clearly specified in suspensor-bearing embryos regardless of the division pattern. Inhibition of auxin response in zygotic embryos causes excessive divisions in suspensor cells and ectopic expression of genes that are normally-restricted to the embryo proper, suggesting that a specific auxin response maintains suspensor cell identity in zygotic embryos (Schlereth et al., 2010; Rademacher et al., 2012).

Conclusion

It is well known that *in vitro*-formed embryos initially show highly variable cell division planes and initially lack clear morphological signs of cell patterning and polar organization (Mordhorst et al., 1997). At which point these structures become embryogenic and how they differentiate are major questions in plant biology, with implications for the normal development of zygotic embryos. We have addressed this question for the first time using embryo and auxin cell fate markers in *B. napus* microspore culture. Our marker data show that, unlike zygotic embryos, irregular cell division patterns or the absence of a suspensor are not necessarily detrimental to microspore embryo development and differentiation. We also show that establishment of the canonical auxin maxima observed in zygotic embryos is not required to establish apical-basal polarity, but rather, is required to establish embryo proper cell fate from the suspensor and to produce functional meristems. If cell fate and patterning during *in vitro* embryogenesis are flexible processes, then why do many Arabidopsis zygotic embryo patterning mutants fail to develop properly? Embryos that develop in microspore culture are autonomous units, while the growth and differentiation of zygotic embryos needs to be coordinated with that of the surrounding seed tissues, the seed coat and the endosperm. Our results raise the possibility that arrest or abortion of many Arabidopsis patterning mutants is due to failure to coordinate the temporal development of the embryo with that of the maternal and filial tissues. Alternatively, many of the Arabidopsis patterning phenotypes might be the result of secondary gene effects on development, rather than the result of defects in cell division patterns per se.

Methods

Plant material and microspore culture

Plants of *Brassica napus* L. cv. Topas lines DH4079 and DH12075 were grown and cultured as described in Supena et al. (2008) with a few modifications. DH12075 microspores were cultured at a higher density (50,000 microspores ml^{-1}), and microspores were incubated for 1 to 3 days, at 32 °C for line DH4079 and at 33.5 °C for line DH12075. After the heat-stress treatment microspore cultures were transferred to 25 °C for further culture. NPA (N-1-naphthylphthalamidic acid, Sigma PS343) was dissolved in DMSO and added to NLN-13 medium at the beginning of the culture. The same volume of DMSO was added to control cultures. The plates were refilled with an equal volume of media after 15 days. For

regeneration of plants from haploid embryos, 4-5 week-old embryos (21 per treatment, three replicates) were transferred to solid B5 medium containing 1% sucrose and cultured at 21 °C under a 16 hour dark: 8 hour light photoperiod.

Reporter lines

The *proGRP:GUS-GFP* reporter line has been previously described (Li et al., 2014). The *proDR5:GFP* construct (*GIIK DR5rev::SV40:33GFP*, (Weijers et al., 2006), and the Arabidopsis *proPIN1:PIN1-GFP* (Benková et al., 2003) and *proPIN7:PIN7-GFP* (Blilou et al., 2005) constructs were transformed to *Agrobacterium tumefaciens* strain C58C1 pMP90 and then to *B. napus* DH12075 (Moloney et al., 1989). All reporter lines showed wild-type phenotypes and embryo yields.

GUS Analysis

GUS staining was performed in GUS staining solution (50 mM sodium phosphate buffer, pH 7.2, containing 10 mM EDTA, 0.1% (v/v) Triton X-100, 2.5 mM potassium ferri- and ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (Duchefa)) for 2 to 4 h at 37°C. Samples were cleared in solution containing water (30 ml): chloral hydrate (80 g): glycerol (10 ml), and observed using differential interference contrast microscopy (Nikon OPTIPHOT).

Pseudo-Schiff-propidium iodide staining (PsPI)

Modified Pseudo-Schiff-propidium iodide staining (PsPI) was performed as described in Truernit et al. (2008) with modifications. Wild-type or GUS-stained samples were fixed as described, but the incubation step in 80% ethanol at 80 ºC was omitted. After fixation, samples were rinsed in water and embedded in 0.9% Sea Plaque Agarose (Duchefa). The samples were then treated overnight with α-amylase (0.3 mg/ml) at 37 °C. After three washes with water, the samples were incubated in 1% periodic acid for 40 minutes and then processed further as described (Truernit et al., 2008).

Confocal microscopy

When indicated, membranes of live material were stained with 10 µg/ml of FM4-64 that was added to the culture medium. Samples were also observed after fixation to allow

combined imaging of GFP and DAPI. Samples were fixed in MTSB buffer, 4% PFA (Sigma) and 0.1% Triton-100 at 4 ºC for at least 24 hours, rinsed several times with MTSB buffer diluted 1:10 in water, counterstained with DAPI (1 µg/ml) or propidium iodide (10 µg/ml), and then mounted in Vectashield (Vector Laboratories).

Fluorescence was observed using a Leica DM5500 confocal microscope. FM4-64 and propidium iodide (PI) were exited with the 532 nm laser line and fluorescence emission detected in the 617 nm and 655 nm light band. GFP was excited with the 488 nm laser line and light emission detected between 510 and 530 nm. DAPI was excited with the 405 nm laser line and detected between 458 and 487 nm. Autofluorescence was detected between 659-784 nm in samples counterstained with DAPI and between 672-713 nm for samples counterstained with PI or FM4-64.

Time lapse imaging

Microspores were embedded at the start of the culture in SeaPlaque agarose (Duchefa) as follows. Freshly isolated microspores were resuspended at a density of 200,000 microspores ml^{-1} in NLN-13 medium. One volume of microspores was mixed with two volumes of a 1:1 mixture of melted 1.8% (w/v) agarose and two-times concentrated NLN-13 medium that was kept at 33 ^{o}C, and then plated in a thin layer onto gridded µ-Dishes (Ibidi) on a pre-warmed electric plate at 33 ^{o}C. The agarose was allowed to solidify for 10 minutes at room temperature and then the dishes were filled with 300 µl of semi-solid 0.45% Sea Plaque agarose in NLN-13. The plates were inverted for imaging.

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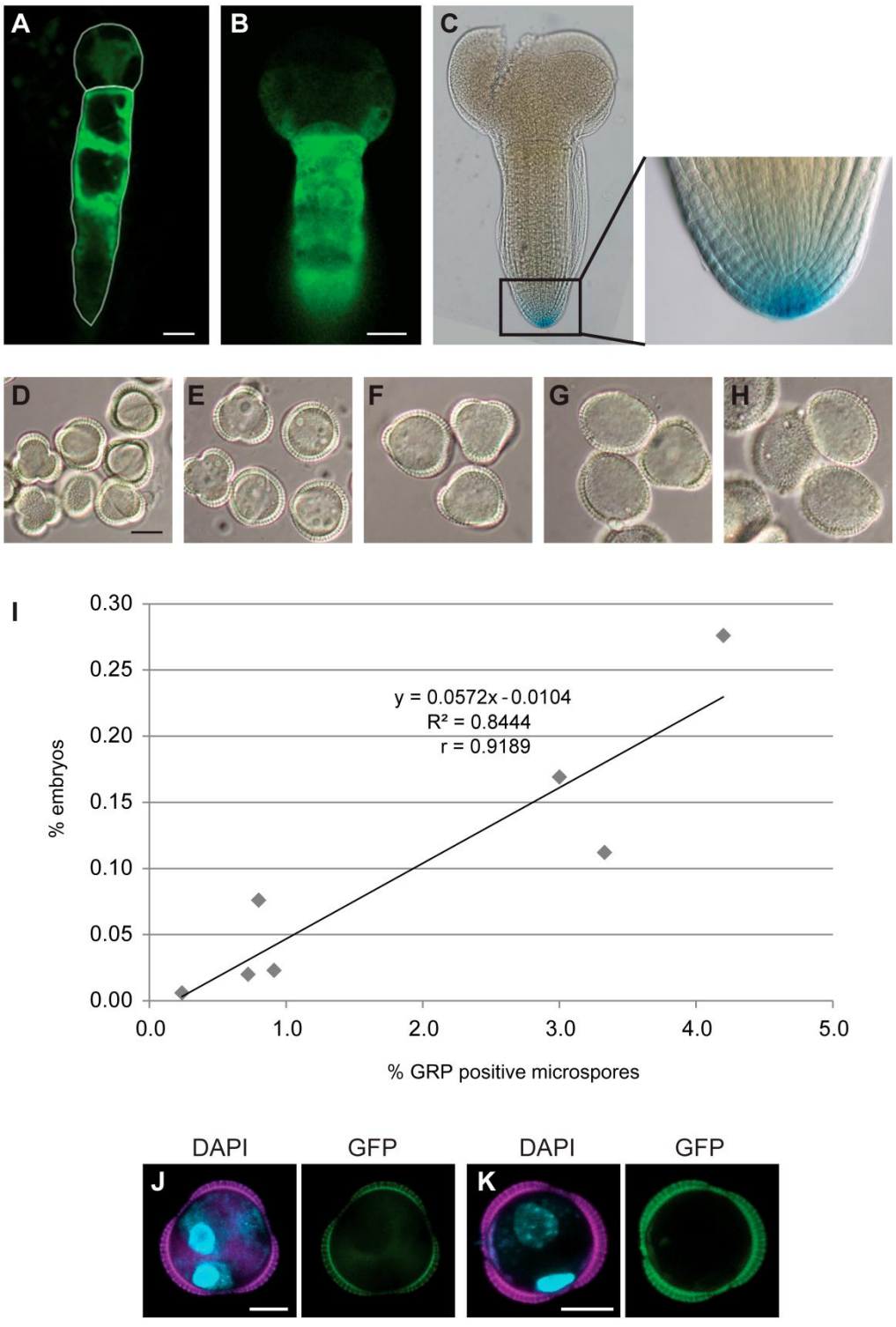
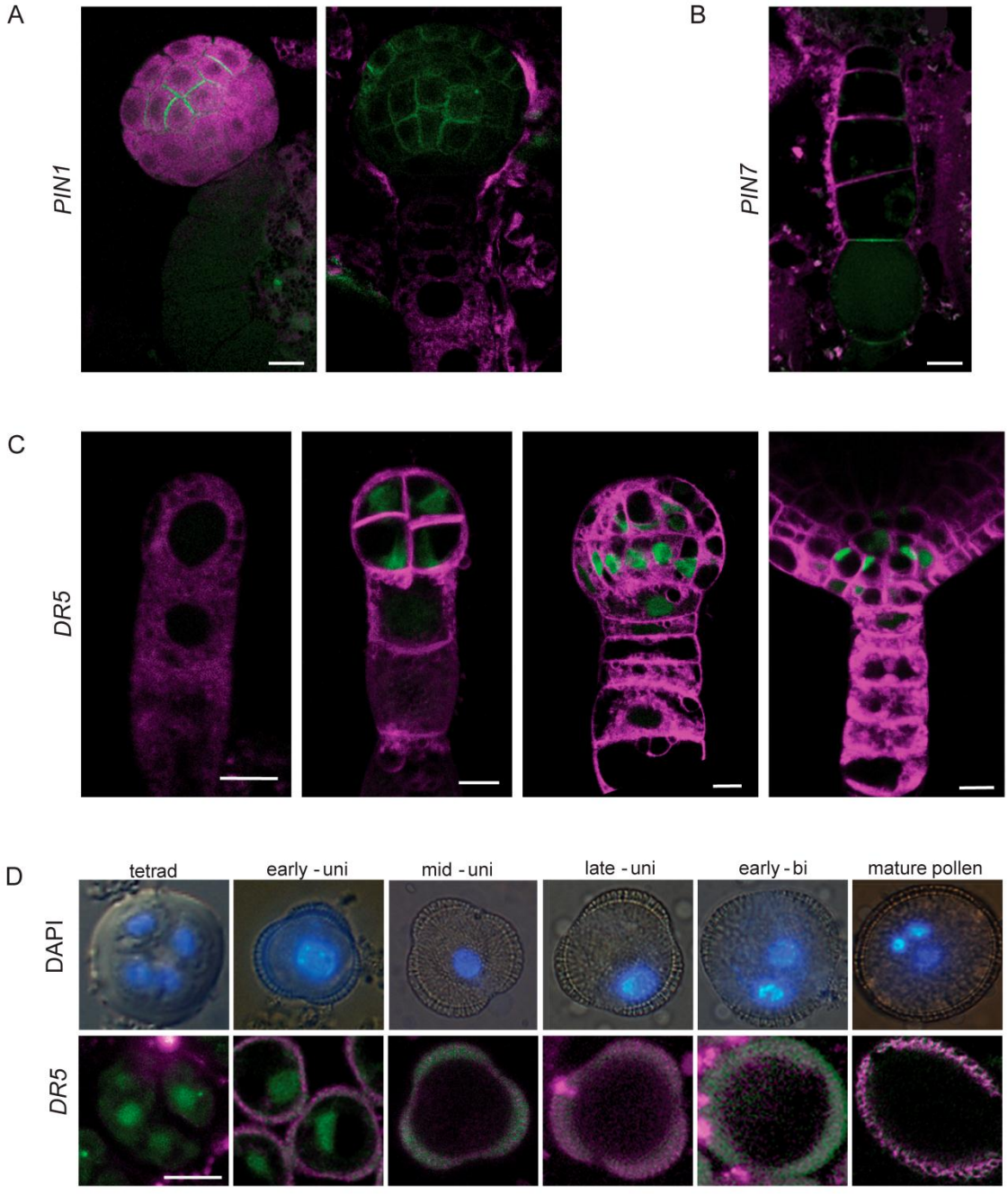
Supplemental data



Supplemental Figure 1. *proGRP:GFP-GUS* expression.

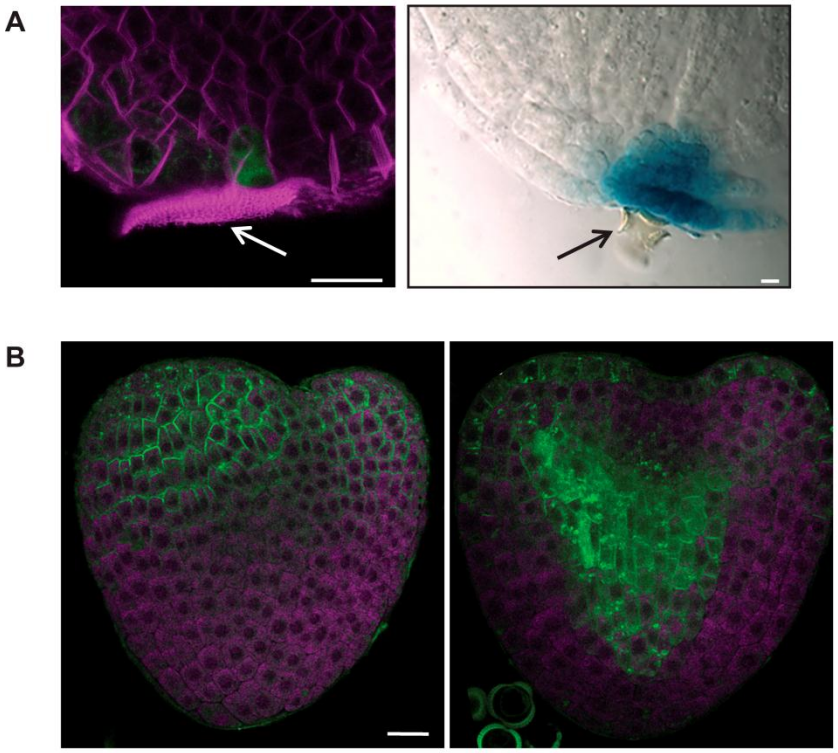
(A-C) Embryo development. **(A)** One-cell stage embryo proper. GFP is expressed in the apical cell and suspensor. **(B)** Globular embryo. GFP is expressed in the suspensor and basal domain of the embryo proper. **(C)** Cotyledon stage embryo. The insert shows GUS staining in the basal tier of the columella root cells. **(D-H)** Pollen development. **(D)** Mid-uninucleate microspores. **(E)** Late-uninucleate microspores. **(F)** Early binucleate pollen.

(G) Trinucleate pollen. **(H)** Mature pollen from dehiscent anthers. (A-H) Bar=10µm. **(I)** Correlation between percentage of *GRP*-positive structures and final embryo yield. **(J-K)** *proGRP:GFP-GUS* expression in microspore culture. **(J)** Symmetrically-divided microspore that does not show *GRP* expression. **(K)** Pollen-like structure that does not show *GRP* expression.



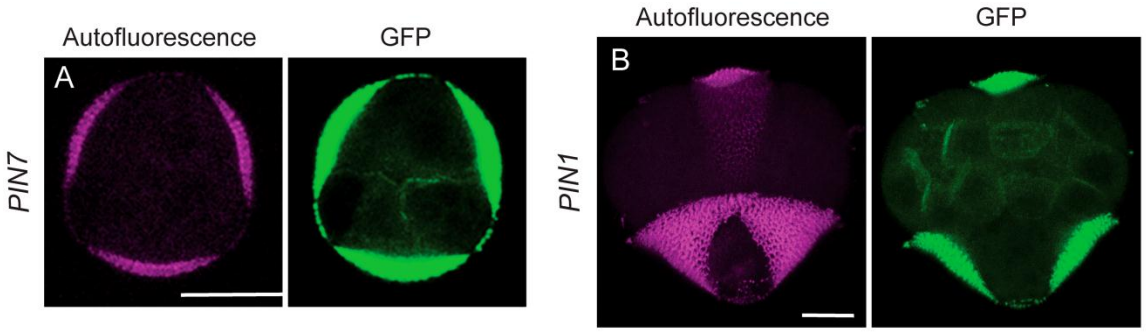
Supplemental Figure 2. Expression of auxin and embryo reporters in *B. napus* embryos and pollen.

(A) PIN1:GFP expression in the embryo proper. **(B)** PIN7:GFP expression in the suspensor. **(C)** *proDR5:GFP* expression in the embryo is observed from the 8-celled stage. **(D)** *proDR5:GFP* is expressed in meiocites and in early uninucleate microspores, but not at later stages of microspore and pollen development. Bar=10µm.



Supplemental Figure 3. *GRP* and *PIN1* expression in microspore embryos after exine rupture.

(A) Expression of *proGRP:GFP-GUS* is associated with the exine remnants. *pro*GRP-driven GFP (left) and GUS (right) expression. Arrow, exine remainings. **(B)** Expression of *proPIN1:PIN1-GFP* in heart stage microspore embryos. PIN1:GFP in the protodermal cells (left) and in the provasculature (right). Bar=20µm.



Supplemental Figure 4. Expression of *proPIN1:PIN1-GFP* and *proPIN7:PIN7-GFP* in two-domain, exine-enclosed embryos.

(A) PIN7:GFP expression. **(B)** PIN1:GFP expression. Autofluorescence (magenta) and GFP (green). Bar=20µm.

References

Bassuner, B.M., Lam, R., Lukowitz, W., and Yeung, E.C. (2007). Auxin and root initiation in somatic embryos of *Arabidopsis*. Plant Cell Rep. **26:** 1-11.

Bayer, M., Nawy, T., Giglione, C., Galli, M., Meinnel, T., and Lukowitz, W. (2009). Paternal control of embryonic patterning in *Arabidopsis thaliana*. Science **323:** 1485-1488.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115:** 591-602.

Berger, F., Taylor, A., and Brownlee, C. (1994). Cell fate determination by the cell wall in early *Fucus* development. Science **263:** 1421-1423.

Berleth, T., and Jurgens, G. (1993). The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. Development **118:** 575-587.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature **433:** 39-44.

Breuninger, H., Rikirsch, E., Hermann, M., Ueda, M., and Laux, T. (2008). Differential expression of *WOX* genes mediates apical-basal axis formation in the *Arabidopsis* embryo. Dev. Cell **14:** 867-876.

Cionini, P.G., Bennici, A., Alpi, A., and D'Amato, F. (1976). Suspensor, gibberellin and in vitro development of *Phaseolus coccineus* embryos. Planta **131:** 115-117.

Custers, J.B.M., Cordewener, J.H.G., Nöllen, Y., Dons, H.J.M., and Campagne, M.M.V. (1994). Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep. **13:** 267-271.

De Smet, I., and Beeckman, T. (2011). Asymmetric cell division in land plants and algae: the driving force for differentiation. Nat. Rev. Mol. Cell Biol. **12:** 177-188.

Eady, C., Lindsey, K., and Twell, D. (1995). The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. Plant Cell **7:** 65-74.

Fan, Z., Armstrong, K.C., and Keller, W.A. (1988). Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma **147:** 191-199.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical–basal axis of *Arabidopsis*. Nature **426:** 147-153.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., and Sandberg, G. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306:** 862-865.

Guillon, F., Larre, C., Petipas, F., Berger, A., Moussawi, J., Rogniaux, H., Santoni, A., Saulnier, L., Jamme, F., Miquel, M., Lepiniec, L., and Dubreucq, B. (2012). A comprehensive overview of grain development in *Brachypodium distachyon* variety Bd21. J. Exp. Bot. **63:** 739-755.

Hadfi, K., Speth, V., and Neuhaus, G. (1998). Auxin-induced developmental patterns in Brassica juncea embryos. Development **125:** 879-887.

Hakman, I., Hallberg, H., and Palovaara, J. (2009). The polar auxin transport inhibitor NPA impairs embryo morphology and increases the expression of an auxin efflux facilitator protein PIN during *Picea abies* somatic embryo development. Tree Physiol. **29:** 483-496.

Hause, B., Vanveenendaal, W.L.H., Hause, G., and Vanlammeren, A.A.M. (1994). Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv. Topas. Botanica Acta **107:** 407-415.

Heisler, M.G., Hamant, O., Krupinski, P., Uyttewaal, M., Ohno, C., Jönsson, H., Traas, J., and Meyerowitz, E.M. (2010). Alignment between PIN1 polarity and microtubule orientation in the shoot apical meristem reveals a tight coupling between morphogenesis and auxin transport. PLoS Biol. **8:** e1000516.

Ilić-Grubor, K., Attree, S.M., and Fowke, L.C. (1998). Comparative morphological study of zygotic and microspore-derived embryos of *Brassica napus* L. as revealed by scanning electron microscopy. Ann. Bot. **82:** 157-165.

Jeong, S., Palmer, T.M., and Lukowitz, W. (2011). The RWP-RK factor *GROUNDED* promotes embryonic polarity by facilitating YODA MAP kinase signaling. Curr. Biol. **21:** 1268-1276.

Johri, B.M., Ambegaokar, K.B., and Srivastava, P.S. (1992). Comparative embryology of angiosperms. (Berlin: Springer).

Joosen, R., Cordewener, J., Supena, E.D.J., Vorst, O., Lammers, M., Maliepaard, C., Zeilmaker, T., Miki, B., America, T., Custers, J., and Boutilier, K. (2007). Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol. **144:** 155-172.

Kaplan, D.R., and Cooke, T.J. (1997). Fundamental concepts in the embryogenesis of dicotyledons: A morphological interpretation of embryo mutants. Plant Cell **9:** 1903-1919.

Korasick, D.A., Enders, T.A., and Strader, L.C. (2013). Auxin biosynthesis and storage forms. J. Exp. Biol. **64:** 2541-2555.

Li, H., Soriano, M., Cordewener, J., Muiño, J.M., Riksen, T., Fukuoka, H., Angenent, G.C., and Boutilier, K. (2014). The histone deacetylase inhibitor Trichostatin A promotes totipotency in the male gametophyte. Plant Cell**:** tpc. 113.116491.

Liu, C.M., Xu, Z.H., and Chua, N.H. (1993). Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell **5:** 621-630.

Lukowitz, W., Roeder, A., Parmenter, D., and Somerville, C. (2004). A MAPKK kinase gene regulates extra-embryonic cell fate in *Arabidopsis*. Cell **116:** 109-119.

Madrid, E.N., and Friedman, W.E. (2009). Female gametophyte and early seed development in *Peperomia* (Piperaceae). Am. J. Bot. **97:** 1-14.

Mayer, U., Buttner, G., and Jurgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development **117:** 149-162.

Moloney, M.M., Walker, J.M., and Sharma, K.K. (1989). High efficiency transformation of *Brassica napus* using Agrobacterium vectors. Plant Cell Rep. **8:** 238-242.

Mordhorst, A.P., Toonen, M.A.J., de Vries, S.C., and Meinke, D. (1997). Plant Embryogenesis. Crit. Rev. Plant Sci. **16:** 535-576.

Nagl, W. (1990). Translocation of putrescine in the ovule, suspensor and embryo of *Phaseolus coccineus*. J. Plant Physiol. **136:** 587-591.

Nakayama, N., Smith, R.S., Mandel, T., Robinson, S., Kimura, S., Boudaoud, A., and Kuhlemeier, C. (2012). Mechanical Regulation of Auxin-Mediated Growth. Curr. Biol. **22:** 1468-1476.

Park, S.K., Howden, R., and Twell, D. (1998). The Arabidopsis thaliana gametophytic mutation gemini pollen1 disrupts microspore polarity, division asymmetry and pollen cell fate. Development **125:** 3789-3799.

Park, S.K., Rahman, D., Oh, S.A., and Twell, D. (2004). *Gemini pollen 2*, a male and female gametophytic cytokinesis defective mutation. Sex. Plant Reprod. **17:** 63-70.

Rademacher, E.H., Lokerse, A.S., Schlereth, A., Llavata-Peris, C.I., Bayer, M., Kientz, M., Freire Rios, A., Borst, J.W., Lukowitz, W., and Jürgens, G. (2012). Different auxin response machineries control distinct cell fates in the early plant embryo. Dev. Cell **22:** 211-222.

Raghavan, V. (1986). Embryogenesis in angiosperms : a developmental and experimental study. (Cambridge: Cambridge University Press).

Raghavan, V. (2004). Role of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2, 4-D. Am. J. Bot. **91:** 1743-1756.

Robert Boisivon, H., Grones, P., Stepanova, A.N., Robles, L.M., Lokerse, A.S., Alonso, J.M., Weijers, D., and Friml, J. (2013). Local auxin sources orient the apical-basal axis in *Arabidopsis* embryos. Curr. Biol. **23:** 2506-2512.

Rosquete, M.R., Barbez, E. and Kleine-Vehn J. (2012). Cellular auxin homeostasis: gatekeeping is housekeeping. Mol. Plant, **5:**772-78.

Rutishauser, A. (1969). Embryology and reproduction biology in the angiosperms. An introduction. (Vienna: Springer Verlag).

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C., and Weisbeek, P. (1994). Embryo origin of the *Arabidopsis* primary root and root meristem initials. Development **120:** 2475-2487.

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E.H., Schmid, M., Jürgens, G., and Weijers, D. (2010). MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature **464:** 913-916.

Schwartz, B.W., Yeung, E.C., and Meinke, D.W. (1994). Disruption of morphogenesis and transformation of the suspensor in abnormal *sus*pensor mutants of *Arabidopsis*. Development **120:** 3235-3245.

Shaw, S.L., and Quatrano, R.S. (1996). The role of targeted secretion in the establishment of cell polarity and the orientation of the division plane in *Fucus* zygotes. Development **122:** 2623-2630.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C.L., Paris, S., Gälweiler, L., Palme, K., and Jürgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science **286:** 316-318.

Sunderland, N., and Evans, L.J. (1980). Multicellular pollen formation in cultured barley anthers: II. THE A, B, AND C PATHWAYS. J. Exp. Bot. **31:** 501-514.

Supena, E.D.J., Winarto, B., Riksen, T., Dubas, E., Van Lammeren, A., Offringa, R., Boutilier, K., and Custers, J. (2008). Regeneration of zygotic-like microspore-derived embryos suggests an important role for the suspensor in early embryo patterning. J. Exp. Bot. **59:** 803-814.

Tang, X., Liu, Y., He, Y., Ma, L., and Sun, M.-x. (2013). Exine dehiscing induces rape microspore polarity, which results in different daughter cell fate and fixes the apical-basal axis of the embryo. J. Exp. Bot. **64:** 215-228.

Telmer, C.A., Newcomb, W., and Simmonds, D.H. (1995). Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma **185:** 106-112.

Touraev, A., Lezin, F., Heberle-Bors, E., and Vicente, O. (1995). Maintenance of gametophytic development after symmetrical division in tobacco microspore culture. Sex. Plant Reprod. **8:** 70-76.

Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemy, J., and Palauqui, J.-C. (2008). High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of phloem development and structure in *Arabidopsis*. Plant Cell **20:** 1494-1503.

Yadegari, R., Paiva, G., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, J.L., Fischer, R.L., Harada, J.J., and Goldberg, R.B. (1994). Cell differentiation and morphogenesis are uncoupled in Arabidopsis *raspberry* embryos. Plant Cell **6:** 1713-1729.

Yeung, E.C., and Meinke, D.W. (1993). Embryogenesis in angiosperms - development of the suspensor. Plant Cell **5:** 1371-1381.

Yeung, E.C., Rahman, M.H., and Thorpe, T.A. (1996). Comparative development of zygotic and microspore-derived embryos in Brassica napus L cv Topas. I. Histodifferentiation. Int. J. Plant Sci. **157:** 27-39.

Tykarska, T. (1976). Rape embryogenesis. I. The proembryo development. Acta Soc. Bot. Pol. **45:** 3-16.

Tykarska, T. (1979). Rape embryogenesis. II. Development of embryo proper. Acta Soc. Bot. Pol. **49:** 391-421.

Ueda, M., Zhang, Z., and Laux, T. (2011). Transcriptional activation of Arabidopsis axis patterning genes WOX8/9 links zygote polarity to embryo development. Dev. Cell **20:** 264-270.

Vernon, D.M., and Meinke, D.W. (1994). Embryogenic transformation of the suspensor in *twin*, a polyembryonic mutant of *Arabidopsis*. Dev. Biol. **165:** 566-573.

Vieten, A., Vanneste, S., Wiśniewska, J., Benková, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development **132:** 4521-4531.

Weijers, D., Schlereth, A., Ehrismann, J.S., Schwank, G., Kientz, M., and Jürgens, G. (2006). Auxin triggers transient local signaling for cell specification in *Arabidopsis* embryogenesis. Dev. Cell **10:** 265-270.

Weijers, D., Sauer, M., Meurette, O., Friml, J., Ljung, K., Sandberg, G., Hooykaas, P., and Offringa, R. (2005). Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in *Arabidopsis*. Plant Cell **17:** 2517-2526.

Wendrich, J.R., and Weijers, D. (2013). The Arabidopsis embryo as a miniature morphogenesis model. New Phytol. **199:** 14-25.

Zaki, M.A.M., and Dickinson, H.G. (1990). Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma **156:** 149-162.

Zhang, J.Z., and Somerville, C.R. (1997). Suspensor-derived polyembryony caused by altered expression of valyl-tRNA synthetase in the *twn2* mutant of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **94:** 7349-7355.

Chapter 6

General Discussion

Epigenetic control of plant developmental transitions in response to environmental cues

Plant development is accompanied by cell fate transitions in response to environmental cues such as temperature, light and nutrient availability. These developmental transitions include the transition from seed development to seed dormancy (seed dormancy), from seed dormancy to seedling (seed germination), from vegetative growth to reproductive growth (flower initiation), from sporophyte development to gametophyte development (gametophyte initiation), and gametophyte development to sporophyte development (embryogenesis) (Hennig and Derkacheva, 2009). Plant developmental growth or transition may arrest or accelerate under different environment cues and plants adopt different strategies such as altered physiological states or gene transcription to respond to different environment cues (Grativol et al., 2012). Epigenetic regulation also plays a major role in response to environmental cues *in planta* (Kim et al., 2010). For example, the *AtCHR12* chromatin-remodeling gene from the SWI/SNF class of chromatin remodellers plays a role in mediating the temporary growth arrest in in response to adverse environmental conditions (Mlynárová et al., 2007). DNA methylation regulates flowering time as a response to low temperature, while a reduced DNA methylation level, achieved by either knocking down DNA metyltransferase *MET1* or *DDM1* promotes flowering (Finnegan et al., 1998).

The occurrence of developmental fate transitions can also be altered when cells and tissues are cultured *in vitro*. During *in vitro* culture, cells can be induced to follow a new developmental pathway (e.g. from a gametophyte directly to an embryo instead of through fertilization) or be held in the same developmental pathway (e.g. zygotic embryo to somatic embryos instead of a seedling). Stress is one of the key treatments that promotes the developmental fate change required for *in vitro* embryo culture (Indrianto et al., 1999; Ikeda-Iwai et al., 2003), but very little is known about how this stress is perceived and translated to promote embryo development (Touraev et al., 1997; Fehér et al., 2003; Zavattieri et al., 2010). In this thesis, we examined the epigenetic regulation of microspore embryogenesis, an example of a stress-induced developmental transition from gametophyte development directly to sporophyte (embryo) development (Chapters 3 and Chapter 4).

Histone acetylation and stress responses

We showed that inhibition of HDAC acetylation induced embryogenic division in microspore culture. Treatment with HDAC inhibitors alone phenocopies the heat-stress

response in microspore culture, but is more efficient when combined with heat stress. Roles for HDAC proteins in mediating biotic and abiotic stress responses have been well documented in Arabidopsis, and include the regulation of cold, osmotic and drought tolerance responses, as well as regulation of pathways associated with the stress hormones, abscisic acid (ABA) and jasmonic acid (Luo et al., 2012a). The histone deacetylases HDA6, HDA19 and HDT3 are involved in stress response and their corresponding mutants are hypersensitive to ABA and salt stress (Chen et al., 2010; Chen and Wu, 2010; Luo et al., 2012b). These mutants display up-regulated expression of the ABA-related genes *ABI1* and *ABI2*, which are associated with increased levels of the activation mark H3K9K14Ac and decreased levels of the repressive mark H3K9me2 in the promoter of both genes (Luo et al., 2012b).

Besides their role in ABA and salt stress responses, HDA6 and HDA19 also play roles in temperature-dependent responses. The *hda6* mutant shows reduced tolerance to acute high temperature stress (Popova et al., 2013). The *hda19* mutant is also sensitive to increased ambient temperature; *hda19* seedlings display normal seedling growth at 24^{o}C, but form pin-like shoots that lack cotyledons when grown at 29^{o}C (Long et al., 2006). This phenotype is probably not a response to temperature stress itself, but rather to increased auxin levels that accompany higher growth temperatures (Szemenyei et al., 2008). We found that *hda6* displays an enhanced, but highly variable efficiency of embryogenic cell division in Arabidopsis microspore culture (Chapter 3), but the link with temperature stress is not clear as donor plants are grown under non-heat-stress conditions(25 ^{o}C).

Stress induces embryo formation around pollen mitosis I (PMI) of microspore/pollen development, but the optimum stage can be later when a stronger stress treatment is applied (Binarova et al., 1997). In Chapter 3, we show that the HDACi TSA can induce microspore embryogenesis at suboptimal temperatures, but has a stronger effect in combination with temperature stress. Also, progressively higher concentrations of TSA are needed to induce embryogenesis from older stages of microspore/pollen. This suggests three things: 1) that HDAC activity is sensitive to (temperature) stress; 2) that overall endogenous HDAC activity or activity of specific HDACs increases as the gametophyte develops; and 3) that a relatively low level of HDAC activity or activity of a specific HDAC at the optimum stage for embryo induction (microspore or early bicellular pollen) provides the competence for an embryogenic response.

Developmental changes in histone acetylation

The changes in histone acetylation status during pollen development, as well as the roles of pollen-expressed HDACs and HATs in this process have not been extensively described. Histone acetylation modification displays dynamic change during pollen development or germination process, and the acetylation level is difference between the vegetative nucleus and generative nucleus (Ribeiro et al., 2009). Studies in *Lilium longiflorum* have shown changes in global patterns of histone acetylation after PMI; the vegetative nucleus of the bicellular pollen displays lower level of H4Ac5 and H4Ac8 acetylation than the generative nucleus and the microspore nucleus. The acetylation level of the vegetative nucleus increases later, during pollen tube growth (Janousek et al., 2000). It has been shown that two HAT proteins *HAM1* and *HAM2* from the MYST subfamily family, which show *in vitro* HAT activity for lysine 5 of histone H4 (H4Ac5), regulate cell division in the male gametophyte. Pollen development arrests before PMI in double *ham1 ham2* mutants (Latrasse et al., 2008), indicating that HATs are essential for pollen development. These studies have tracked overall changes in histone acetylation marks, but no information is available on the roles of HATs or HDACs in controlling locus-specific acetylation marks during pollen development.

There is insufficient information available at this moment to indicate that general HDAC activity or the activity of a specific HDAC changes in the different cells of the male gametophyte during pollen development. Screening of available pollen transcriptome data sets or generation of new data sets could be used to identify potential *HDAC* genes that control gametophytic competence in microspore culture. In addition, chromatin immunoprecipitation with specific acetylated histone antibodies followed by high-throughput sequencing (ChIP-seq) could be used to chart the locus-specific changes in histone acetylation during the course of pollen development and microspore embryo induction. One drawback of these approaches is that the contribution of the vegetative and generative/sperm cells to these expression and acetylation patterns cannot be distinguished. Thus techniques to sort the different gametophytic cells will also be required (Becker et al., 2003; Mitsumoto et al., 2010; Deal and Henikoff, 2011).

A model for HDAC mediated repression of totipotency

Histone acetylation is a dynamic process that involves addition of acetyl groups by HATs and their removal by HDACs. In general histone hyperacetylation at a locus is associated with transcriptional activation and hypoacetylation with transcriptional repression. The static model proposes that HDAC removal of acetyl groups from genes facilitates transcriptional repression, while HAT addition of acetyl groups facilitates transcriptional activation (Shahbazian and Grunstein, 2007). However, the mode of HDAC action in this process is a matter of debate as HDACs have been associated with both transcriptionally active and inactive genes (Kurdistani et al., 2002). Wang et al. (2009) performed a genome-wide analysis of HAT and HDAC chromatin binding in human cell culture and showed that both HATs and HDACs are found at transcriptionally active and inactive genes. They proposed a model in which three transcriptional states, silenced, primed and active were defined in relation to HDAC and HAT activity. Silenced genes are not expressed, lack histone marks and associated HDAC/HAT binding, and are generally associated with the K27me3 repressive mark deposited by polycomb group (PcG) proteins. These genes tend to be lineage-specific and their expression is not rapidly induced by external cues. Primed genes are poised for transcription, but are only activated under the appropriate cellular environment. They promoters of primed genes are associated with H3K4 methylation, low levels of transient HAT and HDAC binding and a low level of acetylation, which prevents Pol II binding. Priming is an epigenetic phenomenon that allows cells to respond quickly to developmental or environmental cues. Actively transcribed genes are associated with high levels of stable HAT and HDAC binding; elongating Pol II recruits HAT to add acetyl groups on the histones during transcription, which are removed by HDAC to reset the chromatin. After treatment with an HDACi the acetylation level of both active genes and primed genes increased, while the acetylation level of silenced genes did not. This indicates that in these cells histone acetylation takes place on both active genes and primed genes (Wang et al., 2009).

A similar model involving HAT-HDAC on acetylation status of primed and active genes can be envisaged for microspore embryo induction (Figure 1A). In this model, during pollen development, stage-specific pollen genes are actively transcribed due to dynamic HDAC and HAT-mediated removal and addition of acetyl groups, respectively, with the balance in favour of acetylation. Upon HDACi treatment, acetylation increases due to the lack of HDAC activity, but the genes remain expressed. In contrast, embryo genes are primed for expression, but are not expressed due to the low level of acetylated histones. HDACi or

stress treatments inhibit HDAC activity of these primed genes, allowing increasing histone acetylation and expression. According to this model pollen gene expression would be initially maintained, but may revert to primed or silenced state after the switch to embryo development. mRNA profiling studies have shown that pollen genes expression is reduced in time as microspore embryos develop (Joosen et al., 2007), although it is still under debate whether some pollen genes remain co-expressed in embryogenic structures (Malik et al., 2007; Li et al., 2014). A static model in which active genes are modified only by HATs and primed genes only by HDACs is also compatible with our data (Figure 1B).

HDAC represses transcription by recruiting other corepressors

We observed that the stage of microspore/pollen development had a negative influence on apical-basal patterning of microspore embryos. Treatment with HDACi improved embryo morphology by promoting apical-basal elongation, but at higher concentrations of specific inhibitors, they also had a negative effect on apical development. Based on similar mutants in Arabidopsis (*gnom*, *mp*, *kuele*, *tpl*, *bdl*) and the known ability of HDACs to interact with the TOPLESS corepressor to repress *ARF* gene transcription, we propose that HDACs are present in a TPL corepressor complex during pollen development, and that this complex can repress basal embryo patterning in microspore embryos. Identification of the major ARF and IAA players in this process, as well as the responsible HDAC protein and its protein-protein partners is required. HDA19 has been shown to interact with other corepressors such as RBR (Rossi et al., 2003), LEUNIG (Gonzalez et al., 2007), TOPLESS-RELATED 1 (TPR1, (Zhu et al., 2010), and the AP2/ERF transcription factor AtERF7 (Song et al., 2005) to repress transcription, thus many potential co-repressors could be involved. However, until now only RBR has been reported to play a role in pollen development (Chen et al., 2009).

In Chapter 4 we showed that *DR5* expression marks a subset of embryogenic microspores. In general, two types of few-celled embryogenic structures develop in microspore culture, compact structures still contained in the exine and structures with loosely connected cells in which the exine has started to break or has completely ruptured. The former is associated with embryo formation, the latter not, although we cannot rule out the possibility that the 'less loose' structures form embryos. Interestingly, these compact structures express *DR5*, while the structures that have burst out of the exine do not express *DR5*. This suggests that activation of an auxin response is associated with successful microspore embryo initiation.

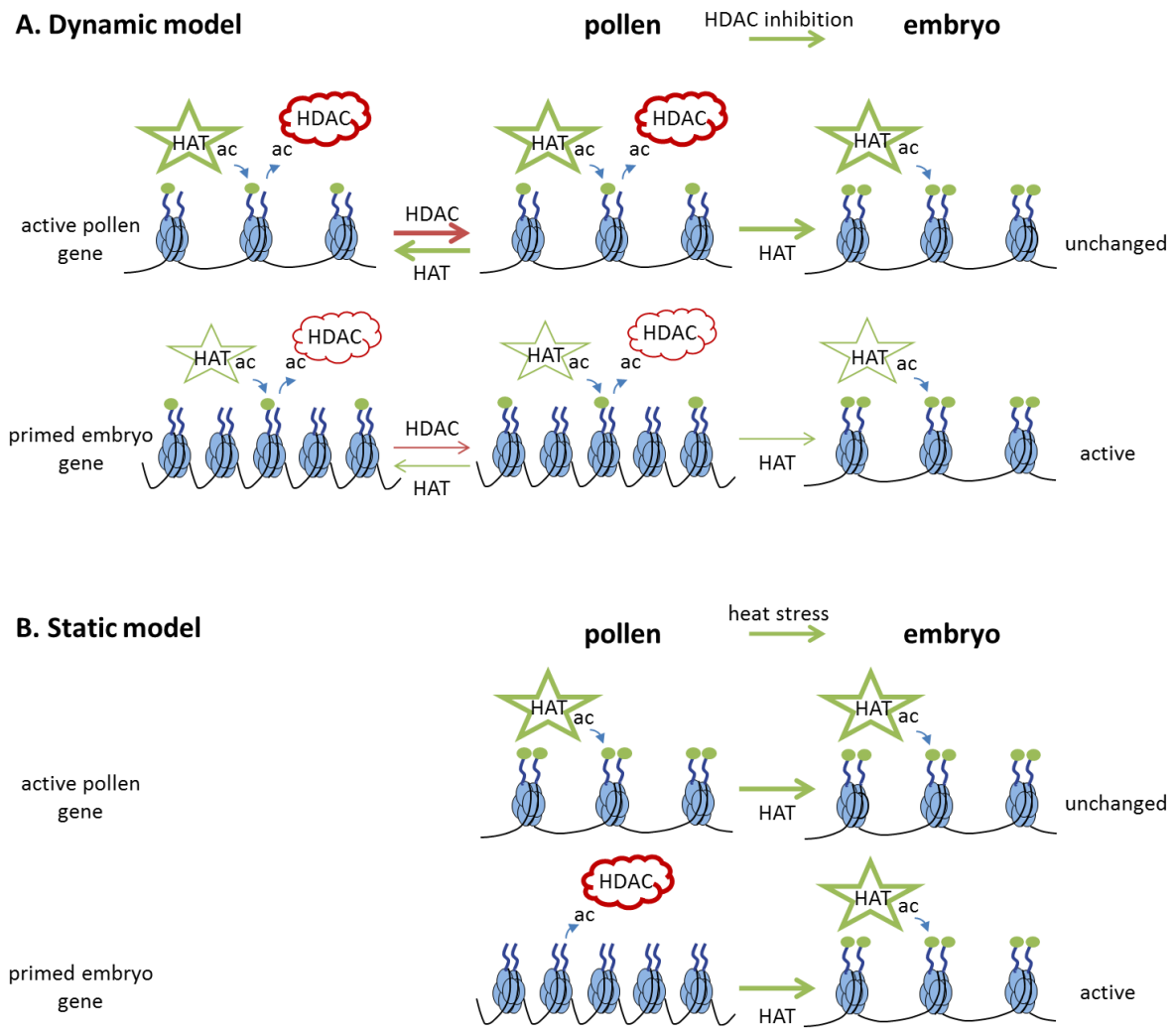


Figure 1. Two models explaining how different histone acetylation states regulate pollen and embryo cell fate. Light blue circles, histone octamers; Green circles: acetyl groups; Black line, DNA strand. The thickness of the HDAC/HAT outline and arrows indicates their relative activity/stability.

Whether this is the same auxin response that regulates embryo patterning needs to be determined, but certainly similarities in the two potential mechanisms in terms of reduced competence for microspore embryogenesis with increasing gametophyte age and increased HDAC-mediated ARF repression with gametophyte age, make this an intriguing possibility. It would therefore be interesting to determine whether a similar HDAC-mediated ARF repression is associated with repression of totipotency in microspore culture. Here again, the availability of Arabidopsis transcriptome data sets and mutant populations could be exploited to answer this question.

The development of callus-like structures and histodifferentiation

A large number of callus-like structures can often be observed in *B. napus* microspore culture, especially in genotypes with a poor embryogenic response. These are characterized by premature rupture of the exine, where the exine either shows multiple breaks, but is still attached or is completely broken. In both cases, the cells are also loosely attached to each other. Classical cell biology studies in *B. napus* suggest that only compact structures (Type I, Chapter 3) form embryos. However, it is not clear whether the less compact, callus-like structures (Types II and IV) develop into embryos. In some HDACi treatments e.g. TSA, a higher proportion of Type I structures is associated with embryo production, while for other inhibitors, e.g. oxamflatin, this type of structure is not correlated with higher embryo yield. Time-lapse imaging of individual microspores/pollen could be used to determine whether callus-like structures can develop into embryos (Daghma et al., 2012).

Recently, Prem et al. (2012) showed suspensor-bearing embryos could be obtained by extended culture at 18 ^{o}C, and that these suspensor-bearing embryos were derived from a subset of cells in loosely connected callus-like clusters This suggests that the loosely-connected structures observed in our culture system are initially programmed to be suspensor-bearing embryos. As with callus-like structures, suspensor embryos burst very early from the exine and do not initially express the *DR5* reporter; *DR5* is only activated once the embryo proper is established from the most distal cell of the suspensor filament. Unlike callus, suspensor growth from the microspore is more controlled, thus callus-like structures may simply represent poorly committed suspensors. However, our marker analysis and microarray analysis suggest that these callus-like structures display embryo identity, and that their transcriptome is highly similar to that of globular-stage embryos. It is not clear if this similarity shows that these structures are actually a poorly developed embryo proper, or reflect the fact that many genes expressed in the embryo proper are also expressed in the suspensor. A detailed comparison of callus-like structures with the transcriptome of microspore embryo suspensors, might provide more insight into the nature of these structures.

Our microarray analysis also showed that callus-like structures show misregulated expression of embryo patterning and auxin signalling genes. It will be necessary in the future to determine how and where these patterning genes are misregulated using of GFP reporters or mRNA *in situ* hybridization. Histodifferentiation begins after the embryo bursts out of the surrounding pollen exine, thus comparison of normal embryos and embryogenic

callus at earlier time points, when these master regulators are not expressed may provide more insight into the different pathways that operate in these two types of structures. However, this approach is complicated by the presence of pollen at earlier time points, which cannot be easily separated from the embryogenic structures.

Brassica and Arabidopsis as model systems

Compared to Arabidopsis, *B. napus* has some limitations for fundamental studies. Firstly, *B. napus* is an amphidiploid of *B. rapa* and *B. oleracea*, each of which contains a triplicated genome relative to Arabidopsis. Although both genome sequences are now publicly available (Wang et al., 2011; Liu et al., 2014), the large number of gene copies makes it difficult to identify the right gene for further analysis e.g. construction of marker lines and to knock-down gene expression. Furthermore, it is difficult and time consuming to transform *Brassica napus*. At least 10 months is required to obtain flowering primary transformations, and not all genotypes are transformable (e.g. a transformation system for the highly embryogenic *B. napus* line DH4079, only became available toward the end of this thesis (Maheshwari et al., 2011).

I attempted to develop an isolated microspore embryo culture system for Arabidopsis by applying hormones and physical stresses, such as cold or heat treatment and osmotic stress, however the viability of isolated microspores/pollen was very low after grinding of flower buds, and the number of microspores that can be isolated is very low. A cut-anther system solved the viability problem, but embryogenic cells were only observed after TSA treatment. The anther wall may provide a protective environment, preventing microspore death, and may also allow culture at higher density. However, anther culture is time-consuming and only the cells that are close to the cut-edge of the anther can be followed. Nonetheless, HDAC-mediated embryogenesis in Arabidopsis anthers is a promising system, at least for the study of embryo induction, as a large number of mutants are available or can be made to study the hypotheses put forward in this thesis.

References

Becker, J.D., Boavida, L.C., Carneiro, J., Haury, M., and Feijó, J.A. (2003). Transcriptional profiling of Arabidopsis tissues reveals the unique characteristics of the pollen transcriptome. Plant Physiol. **133:** 713-725.

Binarova, P., Hause, G., Cenklová, V., Cordewener, J.H., and Campagne, M.L. (1997). A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex. Plant Reprod. **10:** 200-208.

Chen, L.-T., and Wu, K. (2010). Role of histone deacetylases HDA6 and HDA19 in ABA and abiotic stress response. Plant Signal Behav **5:** 1318-1320.

Chen, L.-T., Luo, M., Wang, Y.-Y., and Wu, K. (2010). Involvement of *Arabidopsis* histone deacetylase HDA6 in ABA and salt stress response. J. Exp. Bot. **61:** 3345-3353.

Chen, Z., Hafidh, S., Poh, S.H., Twell, D., and Berger, F. (2009). Proliferation and cell fate establishment during *Arabidopsis* male gametogenesis depends on the Retinoblastoma protein. Proc. Natl. Acad. Sci. USA **106:** 7257-7262.

Daghma, D., Kumlehn, J., Hensel, G., Rutten, T., and Melzer, M. (2012). Time-lapse imaging of the initiation of pollen embryogenesis in barley (*Hordeum vulgare* L.). J. Exp. Bot. **63:** 6017-6021.

Deal, R.B., and Henikoff, S. (2011). The INTACT method for cell type-specific gene expression and chromatin profiling in Arabidopsis thaliana. Nat. Protoc. **6:** 56-68.

Fehér, A., Pasternak, T.P., and Dudits, D. (2003). Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss. Org. **74:** 201-228.

Finnegan, E., Genger, R., Kovac, K., Peacock, W., and Dennis, E. (1998). DNA methylation and the promotion of flowering by vernalization. Proc. Natl. Acad. Sci. USA **95:** 5824-5829.

Gonzalez, D., Bowen, A.J., Carroll, T.S., and Conlan, R.S. (2007). The transcription corepressor LEUNIG interacts with the histone deacetylase HDA19 and mediator components MED14 (SWP) and CDK8 (HEN3) to repress transcription. Mol. Cell. Biol. **27:** 5306-5315.

Grativol, C., Hemerly, A.S., and Ferreira, P.C.G. (2012). Genetic and epigenetic regulation of stress responses in natural plant populations. Biochim. Biophys. Acta - Gene Regul. Mech. **1819:** 176-185.

Hennig, L., and Derkacheva, M. (2009). Diversity of Polycomb group complexes in plants: same rules, different players? Trends Genet. **25:** 414-423.

Ikeda‐Iwai, M., Umehara, M., Satoh, S., and Kamada, H. (2003). Stress‐induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. Plant J. **34:** 107-114.

Indrianto, A., Heberle-Bors, E., and Touraev, A. (1999). Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. Plant Sci. **143:** 71-79.

Janousek, B., Zluvova, J., and Vyskot, B. (2000). Histone H4 acetylation and DNA methylation dynamics during pollen development. Protoplasma **211:** 116-122.

Joosen, R., Cordewener, J., Supena, E.D.J., Vorst, O., Lammers, M., Maliepaard, C., Zeilmaker, T., Miki, B., America, T., and Custers, J. (2007). Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol. **144:** 155-172.

Kim, J.-M., To, T.K., Nishioka, T., and Seki, M. (2010). Chromatin regulation functions in plant abiotic stress responses. Plant, Cell Environ. **33:** 604-611.

Kurdistani, S.K., Robyr, D., Tavazoie, S., and Grunstein, M. (2002). Genome-wide binding map of the histone deacetylase Rpd3 in yeast. Nat. Genet. **31:** 248-254.

Latrasse, D., Benhamed, M., Henry, Y., Domenichini, S., Kim, W., Zhou, D.-X., and Delarue, M. (2008). The MYST histone acetyltransferases are essential for gametophyte development in *Arabidopsis*. BMC Plant Biol. **8:** 121.

Li, H., Soriano, M., Cordewener, J., Muiño, J.M., Riksen, T., Fukuoka, H., Angenent, G.C., and Boutilier, K. (2014). The histone deacetylase inhibitor trichostatin A promotes totipotency in the male gametophyte. Plant Cell**:** tpc. 113.116491.

Liu, S., Liu, Y., Yang, X., Tong, C., Edwards, D., Parkin, I.A., Zhao, M., Ma, J., Yu, J., and Huang, S. (2014). The *Brassica oleracea* genome reveals the asymmetrical evolution of polyploid genomes. Nat. Commun. **5**.

Long, J.A., Ohno, C., Smith, Z.R., and Meyerowitz, E.M. (2006). TOPLESS regulates apical embryonic fate in *Arabidopsis*. Science **312:** 1520-1523.

Luo, M., Liu, X., Singh, P., Cui, Y., Zimmerli, L., and Wu, K. (2012a). Chromatin modifications and remodeling in plant abiotic stress responses. Biochim. Biophys. Acta - Gene Regul. Mech. **1819:** 129-136.

Luo, M., Wang, Y.-Y., Liu, X., Yang, S., Lu, Q., Cui, Y., and Wu, K. (2012b). HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*. J. Exp. Bot. **63:** 3297-3306.

Maheshwari, P., Selvaraj, G., and Kovalchuk, I. (2011). Optimization of *Brassica napus* (canola) explant regeneration for genetic transformation. New Biotechnol. **29:** 144-155.

Malik, M.R., Wang, F., Dirpaul, J.M., Zhou, N., Polowick, P.L., Ferrie, A.M., and Krochko, J.E. (2007). Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol. **144:** 134-154.

Mitsumoto, K., Yabusaki, K., Kobayashi, K., and Aoyagi, H. (2010). Development of a novel real-time pollen-sorting counter using species-specific pollen autofluorescence. Aerobiologia **26:** 99-111.

Mlynárová, L., Nap, J.P., and Bisseling, T. (2007). The SWI/SNF chromatin‐remodeling gene *AtCHR12* mediates temporary growth arrest in *Arabidopsis thaliana* upon perceiving environmental stress. Plant J. **51:** 874-885.

Popova, O.V., Dinh, H., Aufsatz, W., and Jonak, C. (2013). The RdDM pathway is required for basal heat tolerance in *Arabidopsis*. Mol. Plant.

Prem, D., Solís, M.-T., Bárány, I., Rodríguez-Sanz, H., Risueño, M.C., and Testillano, P.S. (2012). A new microspore embryogenesis system under low temperature which mimics zygotic embryogenesis initials, expresses auxin and efficiently regenerates doubled-haploid plants in *Brassica napus*. BMC Plant Biol. **12:** 127.

Ribeiro, T., Viegas, W., and Morais-Cecílio, L. (2009). Epigenetic marks in the mature pollen of *Quercus suber* L.(Fagaceae). Sex. Plant Reprod. **22:** 1-7.

Rossi, V., Locatelli, S., Lanzanova, C., Boniotti, M.B., Varotto, S., Pipal, A., Goralik-Schramel, M., Lusser, A., Gatz, C., and Gutierrez, C. (2003). A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription. Plant Mol. Biol. **51:** 401-413.

Shahbazian, M.D., and Grunstein, M. (2007). Functions of site-specific histone acetylation and deacetylation. Annu. Rev. Biochem. **76:** 75-100.

Song, C.-P., Agarwal, M., Ohta, M., Guo, Y., Halfter, U., Wang, P., and Zhu, J.-K. (2005). Role of an Arabidopsis AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. Plant Cell **17:** 2384-2396.

Szemenyei, H., Hannon, M., and Long, J.A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. Science **319:** 1384-1386.

Touraev, A., Vicente, O., and Heberle-Bors, E. (1997). Initiation of microspore embryogenesis by stress. Trends Plant Sci. **2:** 297-302.

Wang, X., Wang, H., Wang, J., Sun, R., Wu, J., Liu, S., Bai, Y., Mun, J.-H., Bancroft, I., and Cheng, F. (2011). The genome of the mesopolyploid crop species *Brassica rapa*. Nat. Genet. **43:** 1035-1039.

Wang, Z., Zang, C., Cui, K., Schones, D.E., Barski, A., Peng, W., and Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell **138:** 1019-1031.

Zavattieri, M.A., Frederico, A.M., Lima, M., Sabino, R., and Arnholdt-Schmitt, B. (2010). Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron. J. Biotechnol. **13:** 12-13.

Zhu, Z., Xu, F., Zhang, Y., Cheng, Y.T., Wiermer, M., Li, X., and Zhang, Y. (2010). Arabidopsis resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. Proc. Natl. Acad. Sci. USA **107:** 13960-13965.

Summary

Microspore embryogenesis is an expression of plant cell totipotency that leads to the production of haploid embryos. Besides being a widely exploited plant breeding tool, microspore embryogenesis is also a fascinating system that can be used to obtain a deeper mechanistic understanding of plant totipotency. This thesis aims to provide more insight into the process of microspore embryogenesis, from the formation of embryogenic cells to the outgrowth of differentiated embryos.

In **Chapter 1** background information is provided on the various aspects of *Brassica napus* microspore culture and plant development that intersect with the topics that are studied in this thesis. Emphasis is placed on the basic requirements and limitations for successful microspore embryo culture, as well as on the roles of the plant hormone auxin and epigenetic regulation in the development of plant embryos, during both zygotic and *in vitro* embryo development.

Chapter 2 reviews the recent advances that have been made in understanding the developmental and molecular changes that take place during microspore embryogenesis in model systems. The commonly reported cellular changes associated with the establishment of embryo cell fate are summarized and evaluated. The subsequent differentiation of the embryo is also discussed, specifically, what is known about the establishment of polarity, with emphasis on the importance of exine rupture as a positional clue, and the processes that influence meristem maintenance during culture. Finally, the studies on the molecular changes during microspore embryo induction are put into context of male gametophytic development. Overall, the current perspective on microspore embryo initiation presents a landscape in which several routes can lead to the same final destination.

Stress treatments are widely applied to induce embryogenic growth in microspore culture. **Chapter 3** explores the role of histone acetylation status in stress-induced microspore embryogenesis in *Brassica napus*. Inhibition of histone deactylases (HDACs) using the HDAC inhibitor trichostatin A (TSA), phenocopies the heat stress treatment that is normally used to induce embryogenic cell proliferation in *B. napus* microspore culture. Arabidopsis is recalcitrant for haploid embryogenesis, yet treatment with TSA also induced embryogenic cell divisions in this model species. Our observations suggest that the totipotency of the male gametophyte is kept in check by an HDAC-dependent mechanism and that the stress

treatments used to induce haploid embryo development in culture impinge on this HDAC-dependent pathway. The repression of HDACs or HDAC-mediated pathways by stress and the accompanying changes in histone acetylation status could provide a single, common regulation point for the induction of haploid embryogenesis.

Chapter 4 builds on the knowledge developed in Chapter 3 on the role of HDAC proteins in plant totipotency. A wide variety of chemically distinct HDAC inhibitors was evaluated and additional inhibitors that enhance embryogenic cell induction and/or embryo yield were identified. One surprising observation was made during the course of this study: the initial donor microspore/pollen stage affects the quality of the embryo that is formed. In control cultures, embryos from progressively older stages of donor microspores/pollen became progressively compromised in their basal (axis region) region, characterized by a shift from normal embryos with apical (cotyledons) and basal (root) polarity to abnormal embryos with a reduced basal pole and ball-shaped embryos. These abnormal phenotypes could be partially complemented by treatment with HDAC inhibitors, which promoted growth of the basal region of the embryo. Progressive enhancement of embryo basal identity was accompanied by enhanced and broadened expression of the *DR5* auxin response reporter. The embryo phenotypes observed in control and HDAC inhibitor treated microspore cultures are similar to the phenotypes induced by altered expression of the Arabidopsis TOPLESS (TPL)/HDAC19/BODENLOS (BDL) repressor complex, which acts to restrict expression of the AUXIN RESPONSE FACTOR ARF5/MONOPTEROS (MP) to the basal region of the embryo during zygotic embryo development.

To understand why most embryogenic callus failed to develop further, we examined the transcriptome of globular-shaped embryos that have started to histodifferentiate and compared it with embryogenic callus. The transcriptome analysis showed that the expression of many genes that regulate (auxin-related) embryo patterning were downregulated in embryogenic callus compared to globular stage embryos. This result may simply reflect the lack of patterning in these embryos or might indicate a role of auxin-signalling in embryogenic callus formation.

Chapter 5 examines how embryo identity and patterning is established in two *B. napus* microspore embryo pathways, a zygotic-like pathway, characterized by suspensor and then embryo proper formation, and a pathway characterized by initially unorganized structures that lack a suspensor. We specifically asked the question: how can embryo patterning be

established in the absence of an initial asymmetric division and in the absence of a suspensor, two key events in zygotic embryo development. Analysis of embryo fate (*GRP*) and auxin (*PIN1*, *PIN7* and *DR5*) markers showed that embryo fate was established prior to cell division, and independent of subsequent division pattern. The suspensorless embryo program was marked by a transient auxin maximum, followed by establishment of the apical and basal poles at the globular stage, coincident with release of the embryo from the pollen exine. Unlike zygotic embryo development, polar auxin transport (PAT) was not required for embryo initiation or polarity establishment in this system. Suspensor embryos developed in a similar fashion as zygotic embryos, PAT was required for specification of the embryo proper from the suspensor. Haploid embryogenesis therefore follows at least two programs, a PAT-dependent program that requires embryo proper specification from the suspensor, and an alternative PAT-independent program marked by an initial auxin maximum.

In the final chapter, **Chapter 6**, the work presented in this thesis is put in context of the broader plant development field. The epigenetic regulation of developmental transitions that respond to stress and during pollen development are highlighted. A model is provided that histone acetylation levels mediated by HAT and HDAC regulate pollen fate.

Samenvatting

Microspore embryogenese is een vorm van plantencel totipotentie dat leidt tot haploïde embryo's. Behalve dat microspore embryogenese een breed toegepaste veredelingstechniek is, is het ook een fascinerend systeem om plant totipotentie beter te begrijpen. Dit proefschrift verschaft meer inzicht in het proces van microspore embryogenese, vanaf de vorming van embryonale cellen tot de uitgroei van gedifferentieerde embryo's.

In **hoofdstuk 1** wordt achtergrond informatie beschreven over verschillende aspecten van de microspore weefselkweek van *Brassica napus,* in relatie tot de onderwerpen van dit proefschrift. Nadruk wordt gelegd op de basisvoorwaarden voor een succesvolle microspore embryogenese weefselkweek, de rol van het plantenhormoon auxine en de epigenetische regulatie van embryo ontwikkeling in zowel zygotische- als in vitro embryogenese systemen.

Hoofdstuk 2 behandelt de recente doorbraken in het onderzoek aan de moleculaire regulatie en ontwikkeling van microspore embryovorming in modelsystemen. De literatuur over de cellulaire veranderingen die plaats vinden bij het vastleggen van de embryo-identiteit worden besproken. De daaropvolgende differentiatie van het embryo wordt behandeld, met name wat bekend is over de vorming van polariteit en de rol die het open breken van de exine laag daarin heeft. Verder wordt er een vergelijk gemaakt tussen micropsore embrogenese en pollenontwikkeling. Samenvattend is het algemene beeld dat er meerdere wegen zijn die kunnen leiden tot embryo inductie.

Hoofdstuk 3 beschrijft de rol van histon acteylatie in stress-geinduceerde microspore embryogenese in *Brassica napus.* Remming van histon acetylases (HDAC's) met de HDAC-remmer trichostatin A (TSA) levert hetzelfde effect op als hitte stress dat normaal gebruikt wordt voor de inductie van embryonale celgroei in cultures van *B. napus.* Arabidopsis is recalcitrant voor haploide embryogenese, echter behandeling met TSA zorgt ook voor embryonale celdeling in deze modelplant. Onze resultaten geven aan dat de totipotentie van de mannelijke gameet in stand gehouden wordt door een HDAC-afhankelijk mechanisme en dat de stress behandeling die normaal gebruikt wordt voor de inductie van haploide embryo's ingrijpt op de HDAC-afhankelijke route. De remming van HDAC's of HDAC-geregelde routes door stress en de daarmee samenhangende histon acetylatiestatus zou het overkoepelende regulatiemechanisme voor haploide embryogenese kunnen zijn.

Hoofdstuk 4 bouwt voort op de kennis van hoofdstuk 3 over de rol van HDAC remmers op plant totipotentie. Een scala aan chemisch verschillende HDAC remmers is uitgetest en hieruit zijn een aantal extra remmers gevonden die ook in staat zijn embryogenese te stimuleren en embryo aantallen te verhogen. Een opvallende observatie is gedaan tijdens deze studie: het stadium van de microspore/pollen, waarvan uit wordt gegaan, is van belang voor de kwaliteit van de gevormde embryo's. In controle cultures zijn de embryo's die gevormd zijn vanuit oudere micropspore/pollen vooral gestoord in hun basaal gelegen regio, wat tot uiting komt door een verschuiving van normale embryo's met een top (cotylen) en basale (wortel) polariteit naar abnormale embryo's met gereduceerde basale regio en balvormige embryo's. Deze abnormale vormen kunnen gedeeltelijk hersteld worden door een behandeling met HDAC remmers, die de groei van de basale regio van het embryo bevorderen. De graduele verbetering van de basale embryo identiteit ging gepaard met een verhoogde en bredere expressie van de DR5 auxine merker. De embryo's gevormd in controle en met HDAC remmer behandelde cultures zijn vergelijkbaar met embryo's waarin de expressie van het Arabidopsis TOPLESS (TPL)/HDAC19/BODENLOS (BDL) repressor complex veranderd is. Dit complex beperkt de expressie van AUXIN RESPONSE FACTOR ARF5/MONOPTEROS (MP) tot de basale regio van het embryo tijdens zygotische embryo ontwikkeling.

Om te begrijpen waarom de meeste embryonale celclusters niet verder ontwikkelen, hebben we het transcriptoom van deze celclusters vergeleken met die van globulaire embryo's, waarin de weefseldifferentiatie net op gang is gekomen. Deze analyse liet zien dat veel genen die (auxine gerelateerde) patroonvorming in het embryo sturen, waren geremd in de celclusters. Dit resultaat zou simpel te verklaren kunnen zijn door de afwezigheid van patroonvorming in deze celclusters of kan betekenen dat auxin een rol speelt bij de vorming van deze embryonale celclusters.

In **hoofdstuk 5** is de studie beschreven hoe de identiteit en patroonvorming van het embryo tot stand komt in twee verschillende *B. napus* microspore embryogenese routes: the 'zygotische' route die gekenmerkt wordt door de vormig van een suspensor en vervolgens het embryo en de tweede route, waarin in eerste instantie een ongeorganiseerde structuur zonder suspensor gevormd wordt. We hebben specifiek de vraag gesteld hoe patroonvorming tot stand kan komen zonder dat een eerste asymmetrische deling optreedt of een suspensor gevormd wordt. Analyse van de embryo identiteit (GRP) en auxine (*PIN1*,

PIN7 en *DR5)* merkers laat zien dat de identiteit al bepaald is voordat celdeling plaats vindt en onafhankelijk is van de delingspatronen die daarop volgen. Het programma voor een suspensorloos embryo wordt gekarakteriseerd door een tijdelijk auxine maximum, gevolgd door het vastleggen van de top- en basale kanten tijdens het globulaire stadium, dat samen gaat met het verwijderen van de pollen exinelaag om het embryo. Anders dan bij zygotische embryovorming, is polair auxin transport (PAT) niet nodig voor embryo initiatie of polariteit in dit systeem. Suspensor bevattende embryo's ontwikkelen zich op een zelfde manier als zygotische embryo's, waarbij PAT noodzakelijk is voor het specificeren van het embryolichaam van de suspensor. Haploide embryogenese volgt daarom tenminste twee programma's, een PAT afhankelijke route en een alternatieve PAT onafhankelijke route die gekarakteriseerd wordt door een tijdelijk auxine maximum.

In het laatste **hoofdstuk 6** wordt het werk van dit proefschrift in een breder kader van plant ontwikkeling geplaatst. De epigenetische regulatie van veranderingen in pollenontwikkeling, die beïnvloed worden door stress, worden vooral benadrukt. Een model wordt gepostuleerd hoe histon acetylatieniveau's, die ontstaan door HAT en HDAC activiteiten, uiteindelijk bepalen wat er met een pollen gebeurt.

中文摘要

在离体条件下，植物未成熟的花粉（雄性小孢子）能够由配子体发育途径转变为孢子体发育途径，经过胚胎发生途径产生小孢子胚胎，并且能正常萌发成单倍体植株。小孢子诱导产生的单倍体胚胎不仅广泛运用于植物育种中，而且是研究植物胚胎发育的重要模式实验系统。本论文旨在深入理解单倍体胚胎的发生机制，我们以油菜和拟南芥为实验材料，研究方向主要集中在以下两个方面：1、染色质修饰对单倍体胚胎诱导和发育的作用。2、与生长素相关的过程对单倍体胚胎诱导和发育的影响。

第一章以油菜为例，介绍了小孢子胚胎的应用前景和影响小孢子胚胎发生的重要因素，以及生长素和表观遗传因素对合子胚和离体条件下胚胎发育的影响。

第二章对小孢子胚胎研究的最新进展进行综述，主要涉及到离体培养条件下小孢子命运改变及小孢子胚胎形态建成过程中所发生的细胞生物学和分子生物学变化。阐述了细胞分裂模式和胚柄的存在以及花粉壁破裂作为位置信号对小孢子胚胎极性建立的重要性，并从雄配子体发育的角度揭示了小孢子胚胎诱导过程中的分子生物学变化。最后指出运用小孢子胚胎发生体系可寻找胚胎相关基因。

第三章揭示了组蛋白乙酰化状态在小孢子胚胎发生中的作用。胁迫处理能够诱导产生小孢子胚胎，用组蛋白去乙酰化酶抑制剂 TSA 抑制组蛋白乙酰化酶的活性，提高组蛋白乙酰化水平，也可以产生类似胁迫处理的效果，从而诱导小孢子胚性细胞增殖。对于无法诱导小孢子胚胎的拟南芥，经过 TSA 处理也能诱导其小孢子胚性细胞增殖。说明雄配子体的全能性受组蛋白去乙酰化酶控制，胁迫处理诱导产生小孢子胚胎可能也依赖于组蛋白去乙酰化酶途径，遗传分析表明 HDA17 在该过程中起作用。胁迫抑制组蛋白去乙酰化酶或者该酶介导的组蛋白乙酰化状态改变可能是诱导单倍体胚胎产生的调节点。

在第四章，我们评价了一组不同化学结构的组蛋白去乙酰化酶抑制剂对促进小孢子胚胎诱导和发育的作用。组蛋白去乙酰化酶抑制剂包括 SAHA, scriptaid, oxamflatin 和 apicidin 都能够促进胚性细胞增殖或者提高胚胎发生率，但是大多数胚性细胞都不能分化成胚胎。通过转录组分析比较胁迫诱导产生的正常胚胎和组蛋白去乙酰化酶抑制剂诱导产生的胚性细胞团，我们发现它们相关性很大，但是控制胚胎极性建立和分化的关键因子以及生长素和细胞分裂素信号途径相关的基因在胚性细胞团中下调。同时我们发现初始的小孢子或花粉的发育阶段影响最终产生胚胎的质量，组蛋白去乙酰化酶

抑制剂促进较老花粉的胚胎产生率并且促进根芽分生组织的分化，同时增加了的生长素应答。说明乙酰化酶抑制小孢子或花粉的生长素应答，并且与小孢子胚胎形态的建立相关。

油菜早期的合子胚含有胚柄，而且在胚胎发育过程中细胞分裂十分规则。第五章我们探讨了在缺乏胚柄和细胞分裂不规则的情况下，小孢子胚胎是如何建立极性并且发育成正常胚胎的。通过控制胁迫处理的温度，油菜的小孢子胚胎发生可经过两个途径，一个是与合子胚类似的有胚柄的途径，另一个是无胚柄的途径。我们以胚胎特异基因（*GRP*）和生长素相关基因（*DR5, PIN1, PIN7*）为标记基因，揭示了其在小孢子胚胎发育过程中的时空表达模式。研究发现在小孢子胚胎形成过程中，生长素应答发生在第一次细胞分裂之前。在无胚柄的小孢子胚胎产生和极性建立过程中，生长素极性运输不是必需的，花粉壁的破裂可能导致极性的建立；而对有胚柄的小孢子胚胎来说，生长素极性运输是极性建立所必需的。虽然两条途径中小孢子胚胎的细胞分裂不规则，但是后期经历了与合子胚类似的发育过程，说明有规则的细胞分裂对于胚胎命运的和胚胎形态的建立不是必需的。

第六章，我们讨论了该论文的主要工作，指出表观遗传学在植物发育命运转变中的重要作用，特别是组蛋白乙酰化状态的改变对胁迫应答和对植物发育的影响。我们提出模型来解释组蛋白去乙酰化酶如何通过抑制不同基因的转录来控制小孢子的命运。并讨论了组蛋白去乙酰化酶抑制剂在小孢子胚胎诱导方面的应用前景，特别是运用在不能诱导产生单倍体胚胎的重要模式植物和经济作物上。

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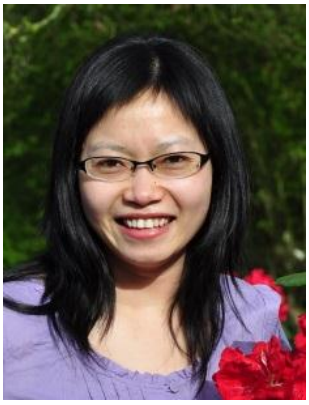
Curriculum vitae



Hui Li was born on the 30th of July 1983 in Zhijiang, Hubei, China. In 2001 she studied in the department of Biotechnology at the Three Gorges University in Yichang, China. After she received her Bachelor degree of Biotechnology in June, 2005, she worked as a technician at the Biotechnology Research Center of the Three Gorges University for one year, where she focused on inducing somatic embryogenesis from endangered plant species in the Three Gorges area. She is interested in plant somatic embryogenesis since then. In 2006, she moved to the Beijing Forestry University to perform her master's study with a specialization in Plant Reproduction and Development, where she studied the regeneration system in *Vitis Vinifera* L. via somatic embryogenesis. She received a Master degree of Botany in 2009. In the same year, she obtained a scholarship from the China Scholarship Council and started her PhD project at the Wageningen University under the supervision of Dr. Kim Boutilier and Prof. Gerco Angenent. Her PhD project is focused on understanding the mechanism of microspore embryogenesis in *Brassica napus* and *Arabidopsis thaliana*. The results are summarized in this thesis. Now she got a position at the Beijing Forestry University as a lecturer and her career will be continued in Plant Sciences.

Publications:

Hui Li, Mercedes Soriano, Jan Cordewener, Jose M. Muiño, Hiroyuki Fukuoka, Gerco C. Angenent and Kim Boutilier. (2014) The histone deacetylase inhibitor trichostatin A promotes totipotency in the male gametophyte. Plant Cell **26**: 195-209.

Hui Li, Mercedes Soriano, José M. Muiño, Tjitske Riksen, Gerco C. Angenent, and Kim Boutilier. The Histone Deacetylase Inhibitors control embryo formation in pollen culture. In preparation.

Hui Li, Fenglan Li, Jingchuan Du, Hai Lu and Zhengquan He. (2008) Somatic embryogenesis and histological analysis from zygotic embryos in *Vitis vinifera* L. 'Moldova'. Forestry Studies in China **10**: 253-258.

Mercedes Soriano, Hui Li and Kim Boutilier. (2013) Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reproduction **26**: 181-196.

Merche Soriano, Hui Li, Cédric Jacquard, Gerco C. Angenent, Joan Krochko, Remko Offringa and Kim Boutilier. (2014) Plasticity in cell division patterns and auxin transport dependency during in vitro embryogenesis in *Brassica napus*. Plant Cell doi: 10.1105/tpc.114.126300.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Hui Li**
Date: **12 September 2014**
Group: **Molecular Biology and PRI-Bioscience, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Development of markers for cell tracking in Brassica embryo cultures	Oct 21, 2009
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter** Microspore embryogenesis: establishment of embryo identity and pattern in culture' , Plant Reprod. 2013 Sep;26(3):181-96. doi: 10.1007/s00497-013-0226-7. Epub 2013 Jul 14.	Jul 2013
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*4.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
Second European Retreat of the PhD Students In Experimental Plant Sciences (Cologne, Germany)	Apr 15-17, 2010
EPS PhD student day, University of Amsterdam, NL	Nov 30, 2012
► **EPS theme symposia**	
2011 EPS theme 1 'Developmental Biology of Plants' , Leiden University	Jan 20, 2011
2012 EPS Theme 1 'Developmental Biology of Plants' , Wageningen University	Jan 19, 2012
2013 EPS theme 1 'Developmental Biology of Plants' , Leiden University	Jan 18, 2013
► **NWO Lunteren days and other National Platforms**	
2009 ALW meeting 'Experimental Plant Sciences' (Lunteren, The Netherlands)	Apr 06-07, 2009
2010 ALW meeting 'Experimental Plant Sciences' (Lunteren, The Netherlands)	Apr 19-20, 2010
2011 ALW meeting 'Experimental Plant Sciences' (Lunteren, The Netherlands)	Apr 04-05, 2011
2012 ALW meeting 'Experimental Plant Sciences' (Lunteren, The Netherlands)	Apr 02-03, 2012
2010 CBSG meeting (Wageningen, The Netherlands)	Sep 28, 2010
2011 CBSG meeting (Wageningen, The Netherlands)	Jan 25, 2011
2012 CBSG meeting (Wageningen, The Netherlands)	Feb 28, 2012
2013 CBSG meeting (Wageningen, The Netherlands)	Feb 13, 2013
CBSG meeting, Proteomics Hotel Projects (Wageningen, The Netherlands)	Dec 14, 2011
► **Seminars (series), workshops and symposia**	
Invited seminar: Following the tracks of cytokinin to uncover principles in plant pattern formation	Oct 29, 2009
Invited seminar: Transcription factories as organizers of the genome: the role of fixed polymerases	Oct 27, 2010
Invited seminar: Lioyd Sumner, Integrated Metabolomics in Medicago truncatula	Aug 30, 2011
Symposia: How to write a world-class paper, Wageningen	Oct 26,2010
Symposia: Advances in life-science Technologies, Wageningen	Nov 25, 2010
Workshop: 5th International PhD school in Plant development (Siena, Italy)	Sep 25-30, 2012
► **Seminar plus**	

►	**International symposia and congresses**	
	Cell-cell communication in plant reproduction (Bath, United Kingdom)	Sep 14-16, 2009
	Molecular Aspects of Plant Development (Vienna, Austria)	Feb 23-26, 2010
	COST action: Plant proteomics in Europe - Systems biology and Omic approaches (Namur, Belgium)	May 05-07, 2010
►	**Presentations**	
	Cell-cell communication in plant reproduction (Bath, United Kingdom) - poster	Sep 14, 2009
	2010 CBSG meeting (Wageningen, The Netherlands) - poster	Sep 28, 2010
	2011 CBSG meeting (Wageningen, The Netherlands) - poster	Jan 25, 2011
	Presentation at Bioscience thematic meeting, Wageningen University	Jan 31, 2012
	2012 CBSG meeting (Wageningen, The Netherlands) - poster	Feb 28, 2012
	5th International PhD school in Plant Development - poster	Sep 25-30, 2012
	Presentation at 2013 EPS theme 1 'Developmental Biology of Plants', Leiden University	Jan 18, 2013
►	**IAB interview**	
	Meeting with a member of the International Advisory Board	Nov 14, 2012
►	**Excursions**	
	Subtotal Scientific Exposure	*18.1 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Postgraduate course 'Bioinformatics-A User's Approach'	Mar 15-19, 2010
	PhD course 'Comparative label-free LC-MS for Quantitative Proteomics'	Apr 21-23, 2010
	PhD course 'Proteomics'	Apr 18-21, 2011
	Postgraduate course 'Transcription Factors and Transcription Regulation'	May 09-11, 2011
	Postgraduate course 'Microscopy and Spectroscopy in Food and Plant Sciences'	May 07-11, 2012
►	**Journal club**	
	Participation in a literature discussion group at Bioscience	2009-2013
►	**Individual research training**	
	Confocal microscopy (private course given by Plant Cell Biology)	Apr 27-28, 2009
	Subtotal In-Depth Studies	*9.8 credits**

4) Personal development		*date*
►	**Skill training courses**	
	PhD competence assessements	Mar 22, 2010
	Advanced course Guide to Scientific Artwork	Nov 04-05, 2010
	EPS Career Day Event: ExPectationS Day, Wageningen	Nov 19, 2010
	Techniques for writing and presenting a scientific paper	Sep 06-09, 2011
	Academic writing	Sep 21, 2011-Feb 06, 2012
	Scientific writing	May 10-Jun 28, 2012
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*7.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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