The Plant Cell, Vol. 26: 195–209, January 2014, www.plantcell.org 

# The Histone Deacetylase Inhibitor Trichostatin A Promotes Totipotency in the Male Gametophyte[W]

**Hui Li,[a] Mercedes Soriano,[a] Jan Cordewener,[a] Jose M. Muiño,[a,b] Tjitske Riksen,[a] Hiroyuki Fukuoka,[c] Gerco C. Angenent,[a,d] and Kim Boutilier[a,1]**

[a] Plant Research International, Bioscience, 6700 AP Wageningen, The Netherlands
[b] Max Planck Institute for Molecular Genetics, D-14195 Berlin, Germany
[c] NARO Institute of Vegetable and Tea Science, Tsu, Mie 514-2392, Japan
[d] Laboratory of Molecular Biology, Wageningen University, 6700 AP Wageningen, The Netherlands

**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 fertilization. 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 postembryonic 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 fertilization 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 unfertilized 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 the application of exogenous growth regulators to abiotic stress. Under the appropriate conditions, the explant resumes cell division and produces differentiated 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 the low percentage of cells that form embryos, have 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

[1] Address correspondence to kim.boutilier@wur.nl.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Kim Boutilier (kim.boutilier@wur.nl).
[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.113.116491

a physical barrier restricting the access of nonhistone 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 remodeling and histone modification (Jiang and Pugh, 2009; Henikoff and Shilatifard, 2011). Chromatin-remodeling proteins use the energy from ATP hydrolysis to remove or reposition nucleosomes (Flaus and Owen-Hughes, 2011), while histone-modifying enzymes chemically modify Lys 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 postembryonic 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-remodeling ATPases (Ogas et al., 1999), members of the Polycomb Group Repressive Complex1 (PRC1) and PRC2, which deposit repressive marks on histones, histone 2A Lys-119 ubiquitination and histone 3 Lys-27 trimethylation, respectively (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 (HDACs), which create a repressive transcriptional state by removing acetyl groups from the Lys residues 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 multilevel dynamic control over cell totipotency.

Changes in chromatin organization 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 article, we examine 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 fertilization. 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 fertilization. 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 differentiated 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 50 years ago in *Datura stromonium* (Guha and Maheshwari, 1964). Since then, many cell biological studies in model species, such as tobacco (*Nicotiana tabacum*), barley (*Hordeum vulgare*), 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 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 4′,6-diamidino-2-phenylindole (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 and Supplemental Data Set 1).

After 2 d of heat stress, microspores/pollen in control cultures arrested, continued gametophyte development, or divided sporophytically. Male gametophyte development in culture followed the same course of development as in the anther (Figures 1A to 1C). The single-celled microspore divided asymmetrically (pollen mitosis [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 (PM II) to produce the two gametes, the sperm cells. In *B. 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,

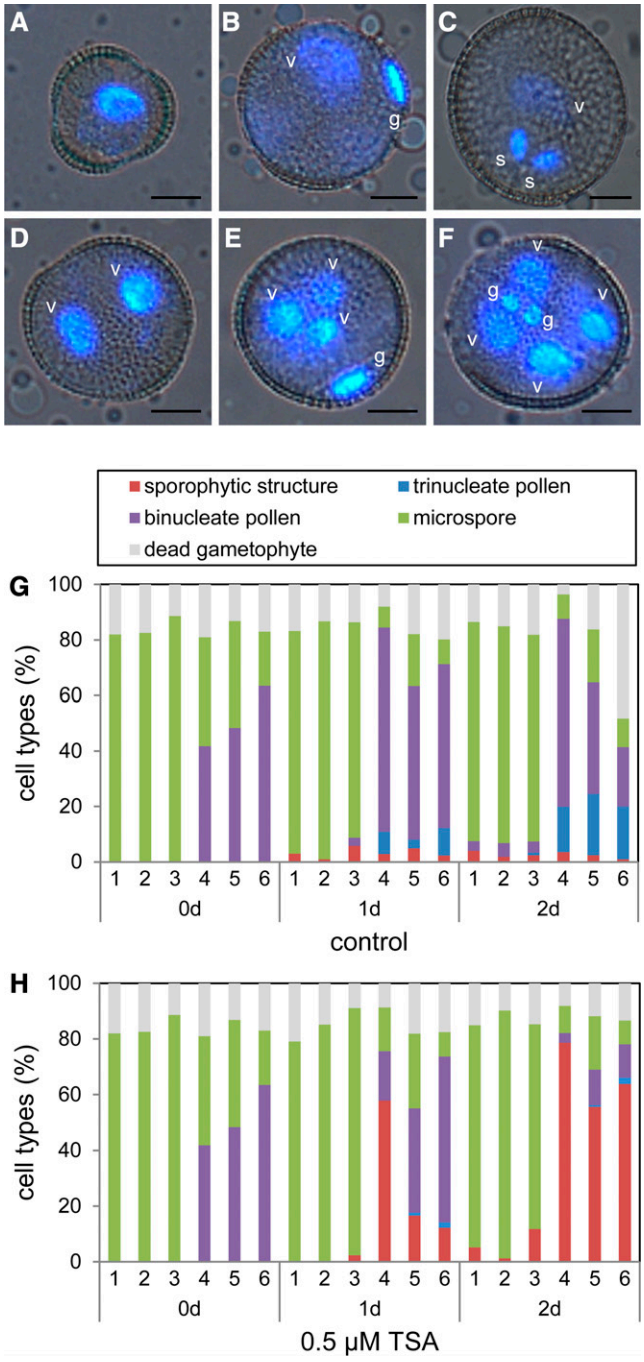


**Figure 1.** Effect of TSA on Early Cell Division Patterns in *B. napus* Microspore Culture.

**(A)** to **(F)** DAPI-stained gametophytic (**[A]** to **[C]**) and sporophytic (**[D]** to **[F]**) structures present in the first 2 d of microspore culture. g, generative (-like) nucleus; s, sperm nucleus; v, vegetative(-like) nucleus. Bars = 10 µm.
**(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)** and **(H)** Percentage of different cell types observed in control **(G)** and TSA-treated **(H)** cultures. The developmental stages of the starting cultures (1 to 6) are ranked from youngest to oldest. The percentages of each structure in control and TSA-treated cultures are shown in Supplemental Data Set 1.

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 gametophyte-like or had died (Figure 1G; Supplemental Data Set 1), as evidenced by the lack of DAPI staining. Up to 6% of the population divided sporophytically within the first 2 d of culture, producing cell clusters with two to six nuclei (Figure 1G; Supplemental Data Set 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 2 d of culture was dramatic, with up to 80% of the population dividing sporophytically (Figure 1H; Supplemental Data Set 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, ~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 suggest that HDAC proteins play a major role in controlling cell cycle progression during male 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, were influenced by the concentration of TSA that was applied to the culture. Therefore, we treated heat-stressed microspores and pollen with a range of TSA concentrations and examined the cultures after 5 and 15 d using DAPI staining to characterize the different multicellular structures that developed.

Four types of sporophytic structures could be distinguished in 5-d-old control cultures (Figures 2A to 2E; Supplemental Figure 2 and Supplemental Data Set 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 5 d 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 they only constituted a small proportion of the population of dividing cells (0.5%). Type II structures were the most abundant structures present in 5-d 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 5-d-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 and Supplemental Data Set 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 10-fold 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 d of culture (Figures 2F and 2G) and were still more abundant in TSA-treated cultures. Type 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 and Supplemental Data Set 1). Compared with 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 differentiated 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 differentiated 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°C divide sporophytically, with the proportion of dividing cells depending on the culture temperature and the stage of male

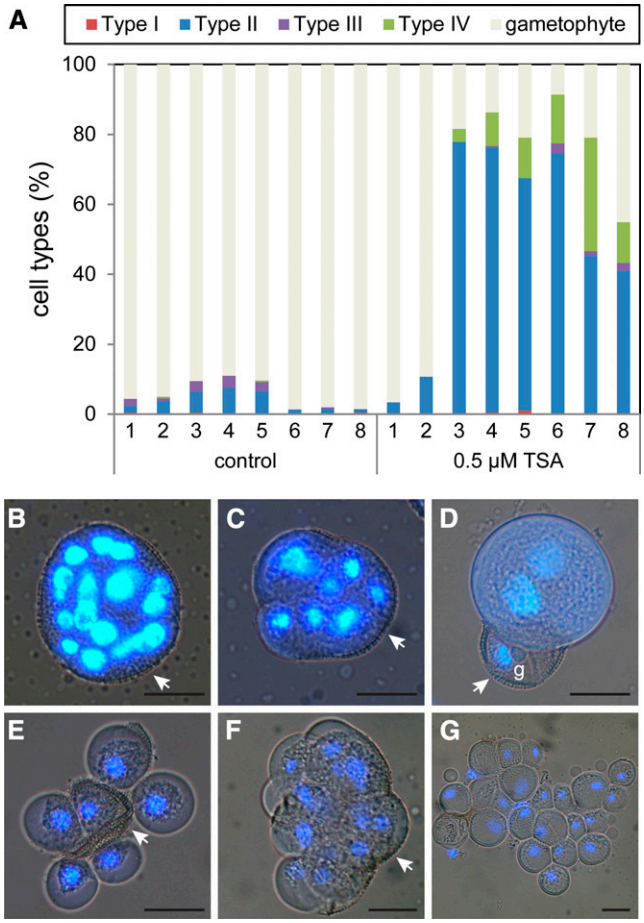


**Figure 2.** Effect of TSA on Sporophytic Growth in *B. napus* Microspore Culture.

**(A)** Percentage of cells that formed pollen or divided sporophytically (types I to IV) after 5 d of microspore culture. The corresponding structures (types I to IV) are shown in **(B)** to **(E)**. The developmental stages of the starting cultures (1 to 8) are ranked from youngest to oldest. The percentages of each structure in control and TSA-treated cultures are shown in Supplemental Data Set 1 and Supplemental Figure 2A.
**(B)** to **(G)** Sporophytic structures after 5 d (**[B]** to **[E]**) and 15 d (**[F]** and **[G]**) of culture. Nuclei are stained with DAPI. Arrows indicate intact **(B)** or broken (**[C]** to **[F]**) exine. g, generative(-like) nucleus. Bars = 20 μm.
**(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.

gametophyte development, but they produce fewer or no embryos compared with 33°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°C as well as a corresponding increase in embryo production at 25°C (Supplemental Figures 3 and 4 and Supplemental Data Set 1). Up to 0.2% embryo production was observed in TSA-treated cultures compared with 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 with cultures grown at 33°C.

Together, our data indicate that treatment with TSA and heat stress mediates 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 were only exposed to a heat stress treatment. They included classical embryogenic structures as well as structures that have been classified as nonembryogenic 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 factor genes, *BABY BOOM* (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 two-cell stage and *GRP* expression was detected from the zygote stage onward (Supplemental Figure 6). Neither gene was expressed during the uninucleate, binucleate, or trinucleate stage of male gametophyte development in the anther (Supplemental Figure 7).

We used the predominantly nuclear localization of the LEC1-GFP fusion to more precisely follow the developmental identity of the different cell types found in microspore cultures within the first 3 d of culture (Figure 3; Supplemental Data Set 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 binucleate or trinucleate pollen-like structures (Figures 3A, 3C, 3E, and 3G). After TSA treatment of heat-stressed microspores, LEC1-GFP was also observed in the same structures as in the control cultures but also in binucleate and trinucleate pollen-like structures (Figures 3B, 3D, 3F, and 3H). 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 (Figures 3D, 3F, and 3I).

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 (Figures 4A and 4B; Supplemental Figure 6), as well as throughout the type II and IV sporophytic structures (Figures 4C, 4D, 4G, and 4H; Supplemental Data Set 1). However, only *LEC1* expression was detected in type III structures (Figures 4E and 4F). 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 heat stress appears to have a stronger effect than 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°C with 0.5 μM TSA (Figures 5A and 5B). 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 that divided sporophytically in TSA-treated Columbia-0 (Col-0) anthers was consistent across experiments (approximately 4%; Supplemental Data Set 1), provided that the anthers were carefully staged, whereas sporophytic divisions were never observed in anthers cultured without TSA (Figure 5C). We examined the 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 PROTEIN4: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 differentiated embryos.

### Behavior 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* to *HDA18*) 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

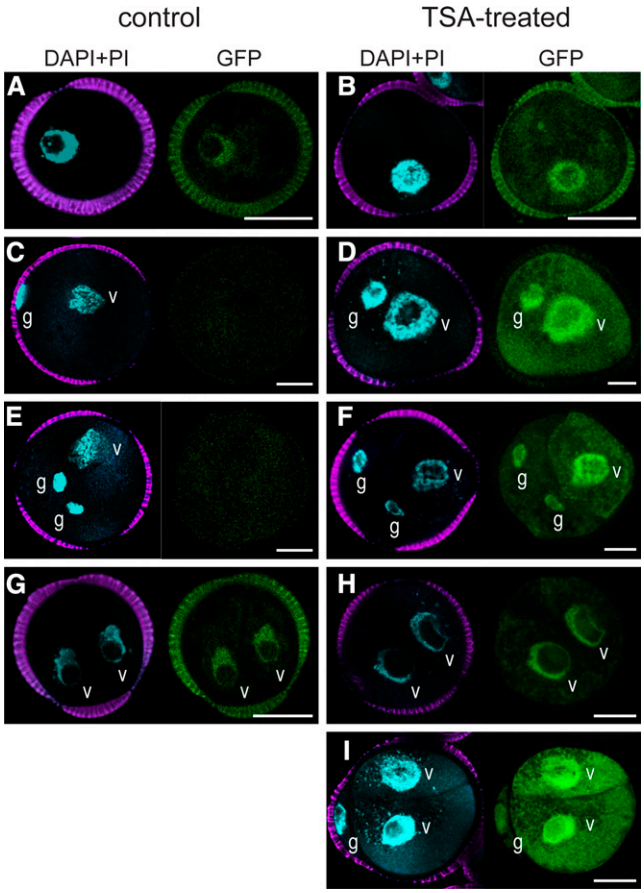


**Figure 3.** TSA Enhances Embryo Marker Expression in *B. napus* Microspore Culture.

Expression is shown for *LEC1:LEC1-GFP* in 2-d-old control (**[A]**, **[C]**, **[E]**, and **[G]**) and TSA-treated (**[B]**, **[D]**, **[F]**, **[H]**, and **[I]**) cultures.
**(A)** and **(B)** Microspore-like structures.
**(C)** and **(D)** Binucleate pollen-like structures.
**(E)** and **(F)** Trinucleate pollen-like structures.
**(G)** to **(I)** Sporophytically divided structures derived from division of a microspore (**[G]** and **[H]**) and a binucleate pollen **(I)**.
For each panel, the image on the left side shows the combined fluorescence from propidium iodide (PI; magenta) and DAPI (blue) staining and the image on the right side shows the GFP fluorescence (green). The green exine in **(A)**, **(B)**, and **(G)** is due to autofluorescence. g, generative-like nucleus; v, vegetative-like nucleus. Bars = 10 μm.

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 (e.g., *hda6* and *hda8*), due to their variable responses 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 Data Set 1). These 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 (*Zea mays*), the Rb protein RETINOBLASTOMA-RELATED1 (RBR1) interacts with an Rpd3-type HDAC, 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 gametophytes (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,

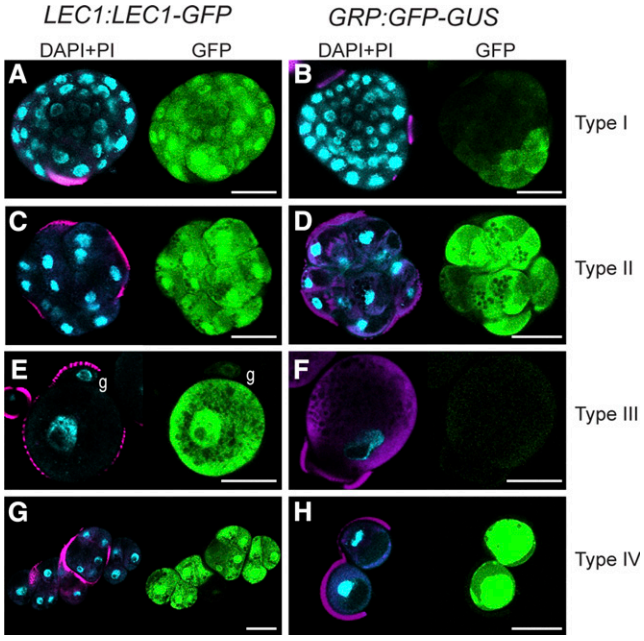


**Figure 4.** Embryo Marker Expression in Sporophytic Structures.

Expression is shown for *LEC1:LEC1-GFP* (**[A]**, **[C]**, **[E]**, and **[G]**) and *GRP:GFP-GUS* (**[B]**, **[D]**, **[F]**, and **[H]**) in 5- to 8 d-old TSA-treated microspore cultures. The same patterns of expression were observed in control cultures.
**(A)** and **(B)** Type I structures.
**(C)** and **(D)** Type II compact callus-like structures.
**(E)** and **(F)** Type III extruded sporophytic structures. g, generative-like nucleus.
**(G)** and **(H)** Type IV loose callus-like structures.
For each panel, the image on the left side shows the combined fluorescence from propidium iodide (PI; magenta) and DAPI (blue) staining and the image on the right side shows the GFP fluorescence (green). Bars = 25 μm.

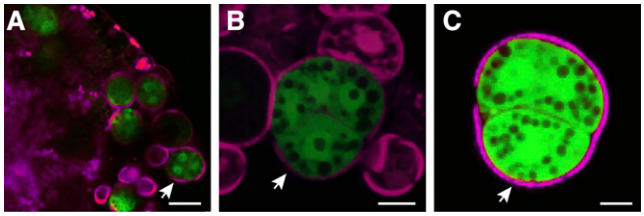


**Figure 5.** TSA Induces Embryogenic Cell Divisions in *Arabidopsis* Male Gametophytes.

Expression of *LEC1:LEC1-GFP* (**[A]** and **[B]**) and *ENODL4:GFP* **(C)** in a type II compact callus-like structure in a TSA-treated anther. The exine (arrows) still surrounds the sporophytic structures. Green indicates GFP, and magenta indicates propidium iodide. All images are from 5-d-old anther cultures. Bars = 25 μm in **(A)** and 10 μm in **(B)** and **(C)**.

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 six nuclei that develop in planta were observed, but at a lower frequency than was reported (Figure 6D; Supplemental Data Set 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 with 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.

### TSA Promotes Histone Acetylation

HDACs deacetylate the Lys residues of both histone and non-histone proteins (Xu et al., 2007). We used an acetylated Lys antibody in combination with protein gel blotting to identify proteins whose acetylation status changed in 8-h heat-stressed, TSA-treated *B. napus* microspore cultures compared with heat-stressed control cultures. We observed increased protein acetylation in low molecular mass proteins in the range of 10 to 25 kD in the TSA-treated cultures compared with 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 most commonly acetylated histones, histones H3 and H4 (Loidl, 2004), during microspore culture using acetylated histone H3 and H4 antibodies. Microspore cultures were started from buds

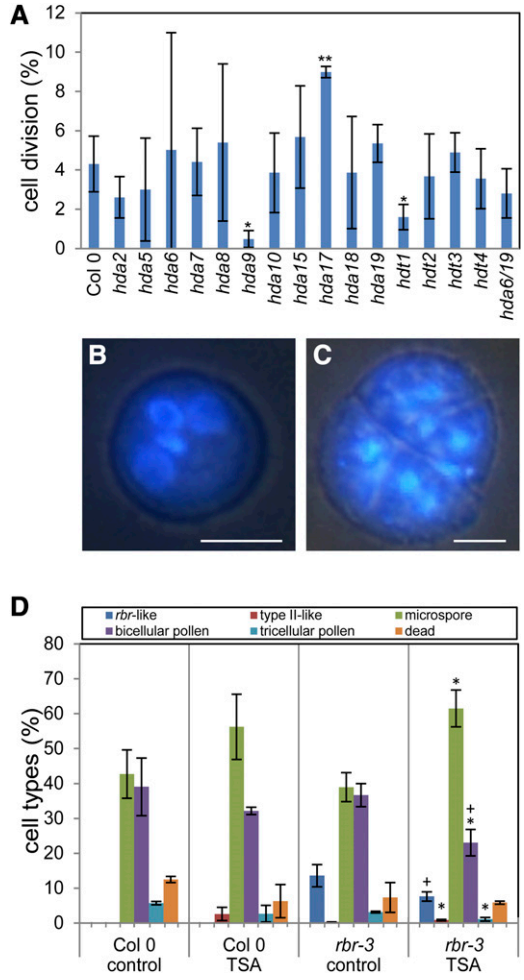


**Figure 6.** Behavior of *hda* and *rbr* Mutants in *Arabidopsis* Anther Culture.

**(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 and **P < 0.01.

**(B)** and **(C)** Multicellular sporophytic structures observed in cultured *rbr-3*/+ anthers. Bars = 10 μm.

**(B)** *rbr*-like multicellular structure with three vegetative-like cells and one generative-like cell.

**(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 responses 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 5 d after the start of culture. The percentage of each structure from Col-0 and mutants in control and TSA-treated cultures is shown in Supplemental Data Set 1.

containing mostly binucleate pollen and placed for 8 h at either 18 or 33°C with or without 0.5 μM TSA. As expected, TSA greatly enhanced sporophytic divisions at 18 and 33°C compared with 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 and 33°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 8 h with TSA, either alone or together with the protein translation inhibitor cycloheximide, to identify primary transcriptional changes. Only a small number of statistically significantly upregulated or downregulated genes were identified (407; Supplemental Figure 8A), and at most a 4-fold change in gene expression was observed between the two treatments and their respective controls (Supplemental Data Set 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 downregulation of a small number of genes (51; Supplemental Figure 6A; Supplemental Data Set 2), more than half of which are pollen-expressed 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 Data Set 2). In contrast to the downregulated gene set, the set of genes that were significantly upregulated after TSA treatment was associated with a wide range of developmental stages and functions (Supplemental Data Set 2). We observed an increase of *LEC1* expression after TSA treatment, but this was not accompanied by major changes in the expression of other early embryo genes or embryo identity regulators (Supplemental Data Set 2). Thus, a large upregulation of embryo gene expression appears to occur later, after 1 to 2 d 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 8 h with TSA (Supplemental Data Set 2). One notable category of upregulated genes includes genes involved in cell wall loosening and degradation (xyloglucan endotransglucosylase/hydrolases), pectin depolymerization and solubilization (polygalacturonases, pectin

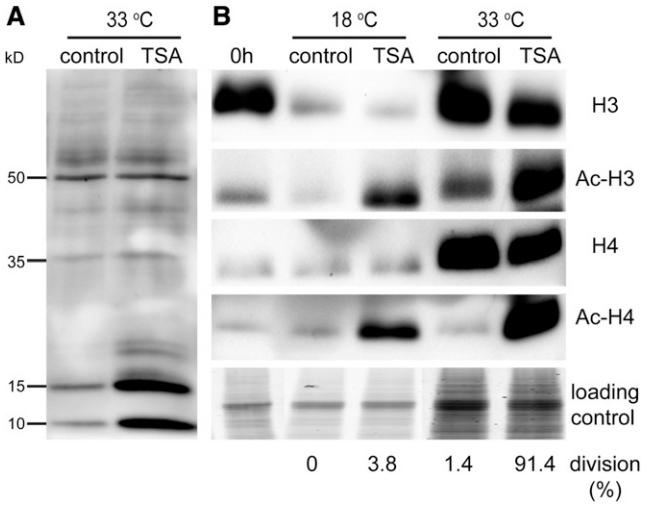


**Figure 7.** TSA Enhances Histone Acetylation.

**(A)** Immunoblot analysis of total acetylated proteins in microspore cultures treated for 8 h with DMSO (control) or TSA. Proteins in the range of 10 to 25 kD are differentially acetylated after TSA treatment compared with the control.
**(B)** Immunoblot of total and acetylated (Ac) histone H3 and H4 in microspore cultures treated for 8 h with DMSO (control) or TSA. The percentages of sporophytic divisions in the different cultures at day 5 are shown under each sample.

polygalacturonase β-subunit protein, pectin methylesterase, pectin esterases, and pectate lyases), and cellulose hydrolysis (*CELLULASE1* [*CEL1*] and *CEL2*). A number of auxin-related genes are also upregulated after TSA treatment (Supplemental Data Set 2). These include two *GH3* genes (*GH3.1* and *DFL1/GH3.6*), which in *Arabidopsis* are known to increase the pool of inactive amino acid–conjugated indole-3-acetic acid (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 the cleavage of indole-3-acetic acid–amino acid conjugates (Rampey et al., 2004). Genes involved in auxin transport through efflux (*PIN1*, *PIN3*, and *PIN7*; Friml et al., 2002) and influx (*AUX1*; Yang et al., 2006) and in auxin signaling (*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 HDAC 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 differentiated embryos and nonviable 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 lower temperature (18 or 25°C) than at higher temperature (33°C). In a similar fashion, a more severe (41°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 is the E2F transcription factors, which dimerize with DP proteins to activate the 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 the 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 dimerization 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*, was 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 maintain the mitotic cycle in roots, preventing endoreduplication (Dewitte et al., 2007). These results suggest that HDAC inhibition induces cell proliferation through the 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, therefore, is different from that observed after the 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 reactivation of cell division in the vegetative cell; however, our examination of embryo reporter lines and microarray analysis showed that embryo gene expression was activated prior to cell division. This observation is striking, as the establishment of new cell fates in both plants and animals usually requires an asymmetric cell division (reviewed in De Smet and Beeckman, 2011) or the formation of transit-amplifying (meristem) cells (reviewed in Sablowski, 2011).

The expression of embryo identity genes prior to sporophytic division raises the question of whether their expression is sufficient to drive cell division toward totipotent growth or 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 5 d after the start of culture and also have 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 coexpression 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 differentiated 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 5 to 6 d 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 2-cell 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 to IV structures are much larger than the compact structures that form differentiated embryos, but it is not clear whether this increased size causes exine rupture or cell expansion occurs after rupture, for example, in response to the 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 the expression of genes encoding cell wall mobilization enzymes, particularly those involved in the mobilization of cellulose and pectin. One possibility is that the composition of the cell wall or the connection between cell walls in type II to IV structures is altered, preventing the proper cell-to-cell communication required for differentiation.

We also observed an increase in the expression of genes involved in the auxin pathway. The role of endogenous auxin and auxin signaling in haploid embryo induction has not been examined, but exogenous auxin is not required to induce microspore embryogenesis in *B. napus*. By 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 the removal of 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, are associated with the induction of callus-like structures or compact, differentiated 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 the 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 is a splice variant of HDAC9 that lacks the HDAC domain. The MEF2-interacting transcription repressor 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 the formation of HDAC protein dimers (Luo et al., 2012) between HDA17 and one or more *Arabidopsis* HDAC proteins.

In *Arabidopsis* seedlings, TSA treatment induces postgermination 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* mutant nor the double *hda6 hda19* mutant 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* 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°C for 20 h and subsequently transferred to 25°C. Noninduced microspore cultures were cultured continuously at 25 or 18°C. 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 h 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°C.

*Arabidopsis thaliana* flower buds at stage 11 were collected for anther culture. Flower buds were surface-sterilized in 2% bleach for 10 min and 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°C for 20 h in the dark. The medium was then replaced by fresh NLN-13 medium by pipetting gently, and the cultures were incubated at 25°C for an additional 4 d. Free and loosely attached microspores were collected and stained with DAPI. *Arabidopsis hda* T-DNA insertion lines were obtained from the 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 protein (Gray-Mitsumune and Matton, 2006) and is highly similar to a *B. napus* Gly/Pro-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. *ENODL4* was identified as an early embryogenesis–expressed gene from *B. napus* microspore culture (Japanese patent number 3593565; Hiroyuki Fukuoka, Tatsuya Ikeda, and Hiroshi Yano, NARO). A 1035-bp fragment of the promoter of *ENODL4* (GenBank accession number 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 the 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 DAPI (1.25 μg/mL) according to Custers (2003) using a Zeiss Axioskop epifluorescence microscope with filter set 02. Approximately 200 microspores or multicellular clusters were counted for each sample. Confocal laser scanning microscopy was performed on a Leica DM5500 Q microscope. The GFP was excited with an argon laser line at 488 nm and detected with a 505- to 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- to 660-nm emission filter. DAPI was excited at 405 nm and detected with a 440- to 500-nm emission filter. The optical slices were median filtered with Leica LAS AF software. *Arabidopsis* anthers were cleared in water: chloral hydrate:glycerol (3:8:1) solution for 10 min and then observed by differential interference contrast 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°C for 30 s and 30 cycles comprising 98°C for 5 s, 60°C for 30 s, followed by 72°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 the 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°C for 8 h with either TSA or TSA plus cycloheximide (Sigma-Aldrich), both dissolved in DMSO. DMSO and cycloheximide was used as mock treatment for the TSA and TSA + cycloheximide treatments, respectively. The samples were harvested by centrifugation for total RNA isolation, as described above. One microgram of total RNA from each sample was sent to the NASC Affymetrix Service (http://affymetrix.Arabidopsis.info/) for hybridization to the Affymetrix Brassica Exon 1.0 ST GeneChip. Probe annotations were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The identifier for the annotation is GPL10733. The expression data were subjected to normalization using the robust multiarray average method from the *affy* Bioconductor package. $Log_2$-transformed expression values were identified as differentially expressed using Student's *t* test. Multiple hypothesis testing correction was done using the method of Holm (1979) implemented in the multtest Bioconductor package. Mapman (Thimm et al., 2004) was used to identify functional categories of differentially expressed genes. The microarray data have been deposited to the Gene Expression Omnibus database (GSE49070).

### Immunochemistry

Freshly isolated microspores and microspores cultured for 8 h 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 on a Midget 12.5% SDS-PAGE gel under reducing conditions. After transfer of the proteins to a polyvinylidene difluoride membrane and blocking with 5% milk powder in PBS and 0.1% Tween 20, the blots were incubated for 2 h with primary antibody (1:2000 dilution). The primary antibodies used in this study were as follows: anti-acetyl-Lys (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 horseradish peroxidase antibody (Sigma) was used in a 1:2000 dilution, and signals were detected using enhanced chemiluminescence (SuperSignal West Femto Chemiluminescent Substrate; Pierce).

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ENOD4L*, AB836663, AB098076; *LEC1*, At1g21970; *GRP*, At2g30560.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Effect of the Duration of TSA Treatment on Sporophytic Cell Division in *B. napus* Microspore Culture.

**Supplemental Figure 2.** Effect of TSA on Cell Fate and Embryo Formation in *B. napus* Microspore Culture at 33°C.

**Supplemental Figure 3.** Effect of TSA on Cell Fate and Embryo Formation in *B. napus* Microspore Culture at 25°C.

**Supplemental Figure 4.** Effect of TSA on Cell Fate and Embryo Formation in *B. napus* Microspore Culture at 18°C.

**Supplemental Figure 5.** RT-PCR Analysis of Cell Fate Marker Expression in *B. napus* Microspore Culture.

**Supplemental Figure 6.** Expression of the *LEC1*, *GRP*, and *ENOD4L* Reporter Lines in Zygotic Embryos.

**Supplemental Figure 7.** Embryo Reporters Are Not Expressed during Pollen Development in Planta.

**Supplemental Figure 8.** Microarray Analysis and Validation.

**Supplemental Figure 9.** TSA-Downregulated Genes Are Preferentially Expressed in Pollen and Pollen Tubes.

**Supplemental Table 1.** Summary of the Expression Patterns of the *LEC:LEC1-GFP* and *GRP:GFP-GUS* Reporters in *B. napus* Microspore Culture.

**Supplemental Table 2.** DNA Primers Used for Genotyping *hda* T-DNA Insertion Lines.

**Supplemental Table 3.** DNA Primers Used for RT-PCR and Microarray Validation.

**Supplemental Data Set 1.** Developmental Pathways Found in Control and TSA-Treated Cultures.

**Supplemental Data Set 2.** Significantly Differentially Regulated Genes Observed by Microarray Analysis.

## ACKNOWLEDGMENTS

We thank Mieke Weemen (Plant Research International) for technical assistance, Ginette Seguin Schwartz (Agriculture and Agri-Food Canada) for the DH12075 seeds, and Zhong Chen (Temasek Life Sciences Laboratory) for the *rbr-3*/+ seeds. This work was funded by the Centre for BioSystems Genomics (grants to K.B.) and the China Scholarship Council (fellowship to H.L.).

## AUTHOR CONTRIBUTIONS

H.L., M.S., K.B., and G.C.A. designed the research. H.L., M.S., J.C., H.F., and T.R. performed the research. J.M.M. analyzed the data. H.L., M.S., G.C.A., and K.B. wrote the article.

Received July 29, 2013; revised December 19, 2013; accepted January 9, 2014; published January 24, 2014.

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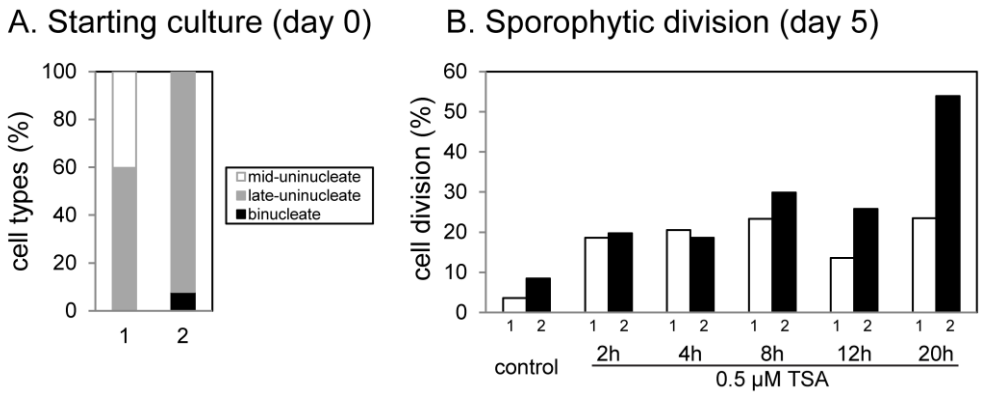
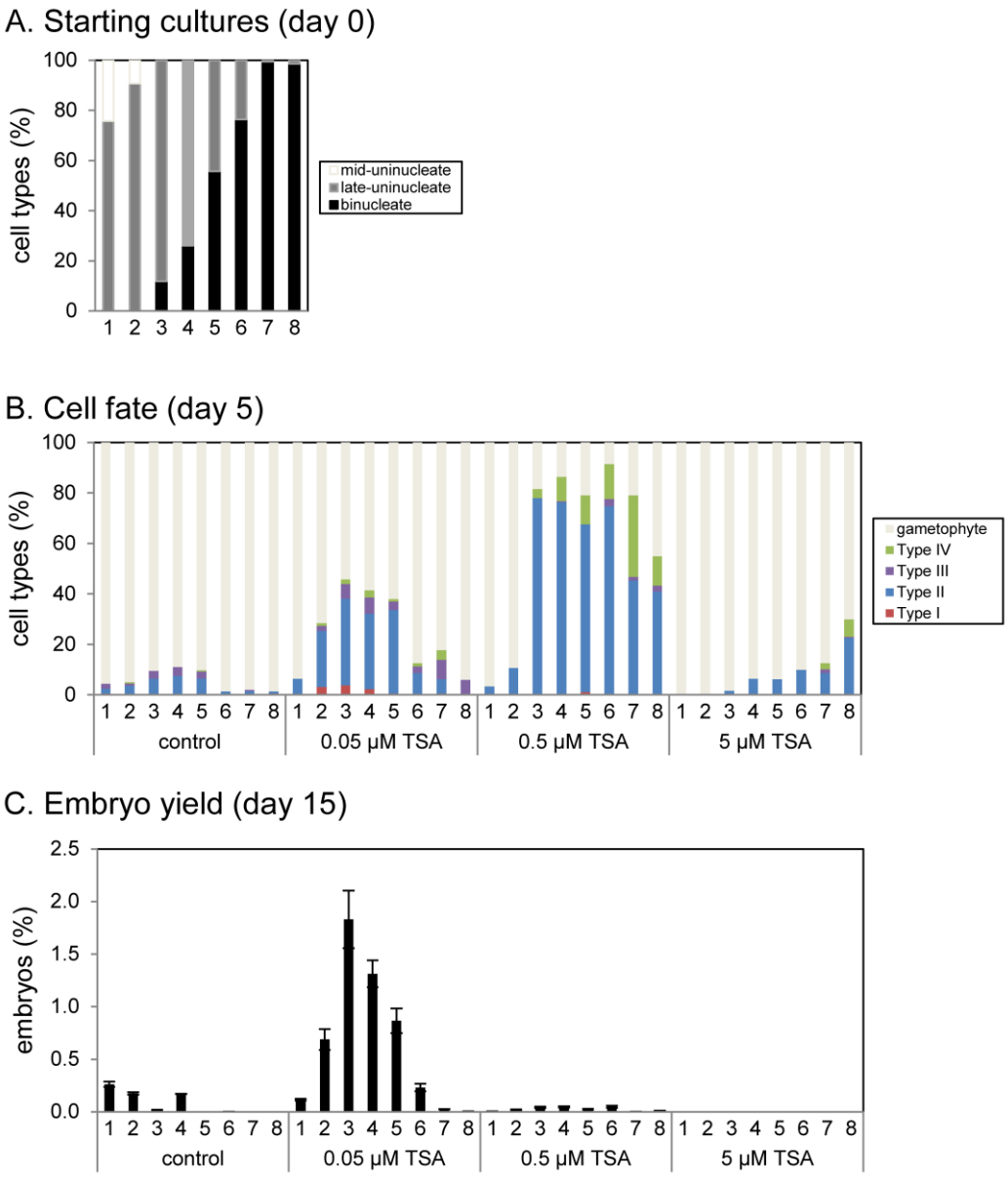



**Supplemental Figure 1.** Effect of the duration of TSA treatment on sporophytic cell division in *B. napus* microspore culture at 33 $^{o}$C.

**(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**).



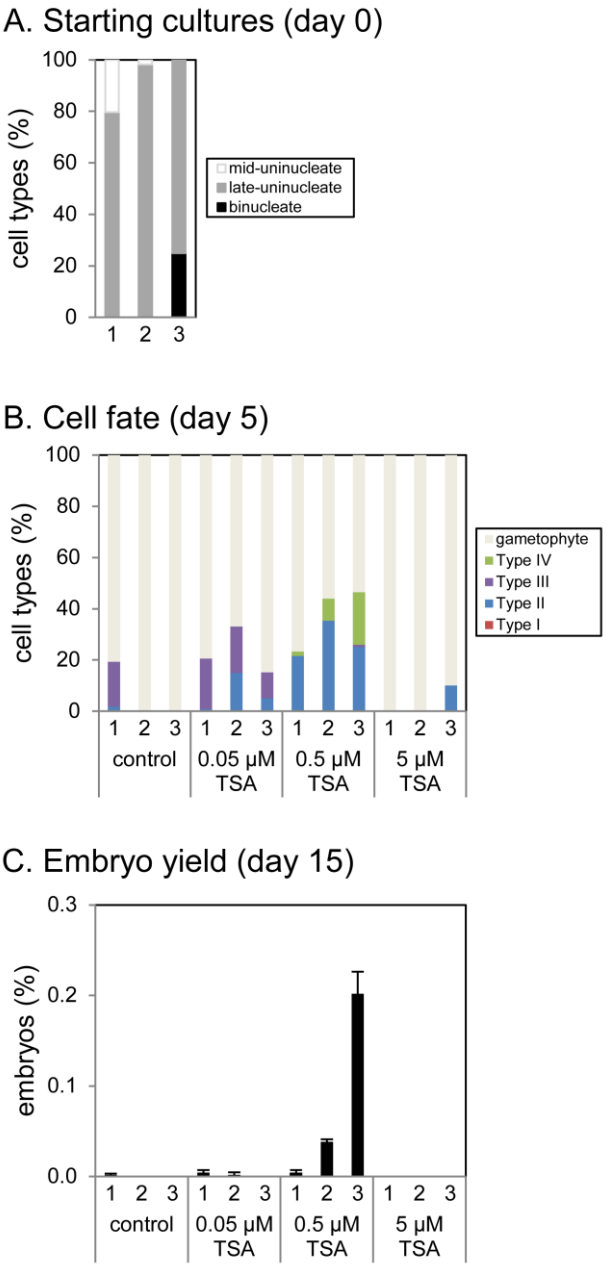

**Supplemental Figure 2**. Effect of TSA on cell fate and embryo formation in *B. napus* microspore culture at 33 $^{o}$C.

**(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.



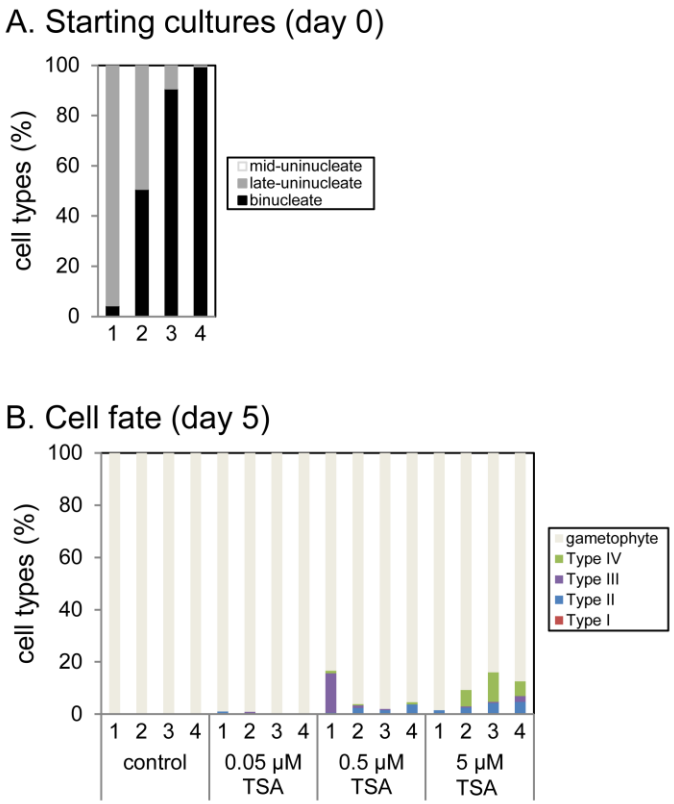

**Supplemental Figure 3**. Effect of TSA on cell fate and embryo formation in *B. napus* microspore culture at 25 $^{o}$C.

**(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.



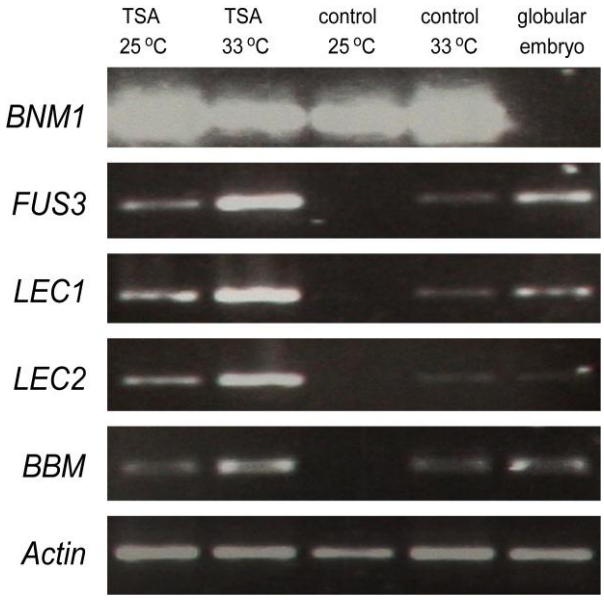

**Supplemental Figure 4**. Effect of TSA on cell fate and embryo formation in *B. napus* microspore culture at 18 $^{o}$C.

**(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.

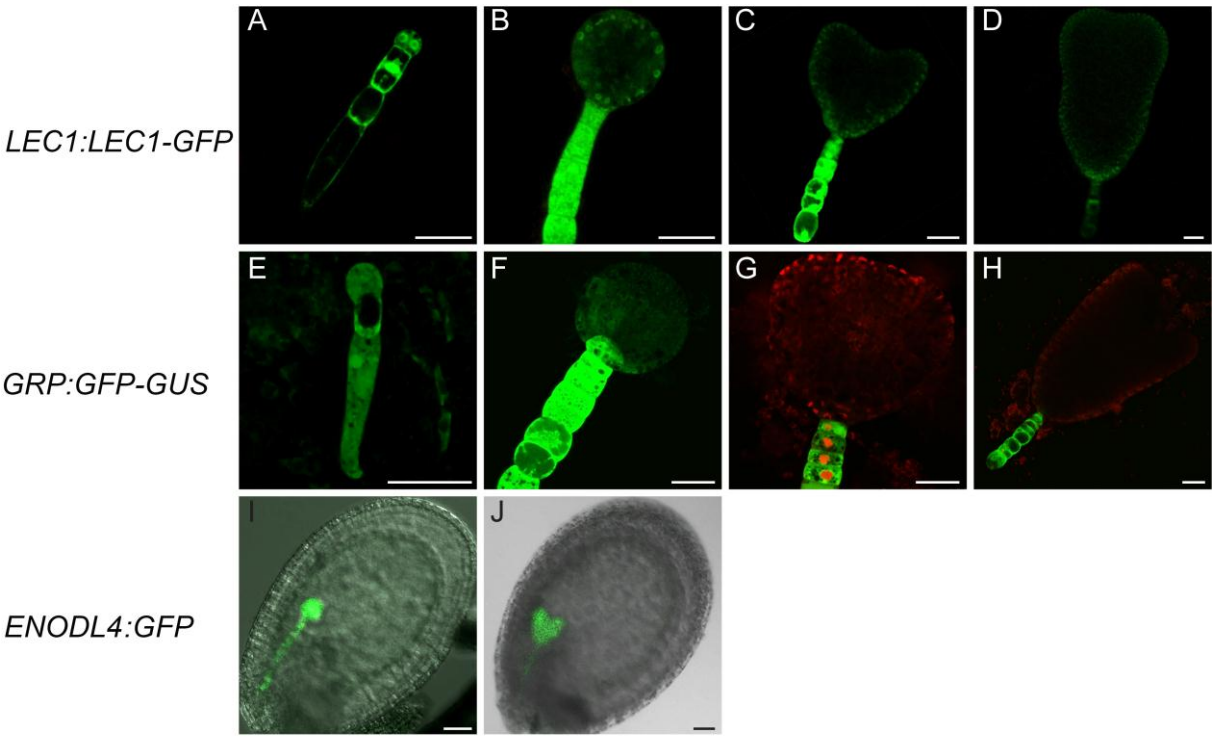
Histodifferentiated embryos did not develop in control and TSA-treated samples that were cultured at 18 $^{o}$C.

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.** RT-PCR analysis of cell fate marker expression 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 $^{o}$C and 25 $^{o}$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 3.




**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.

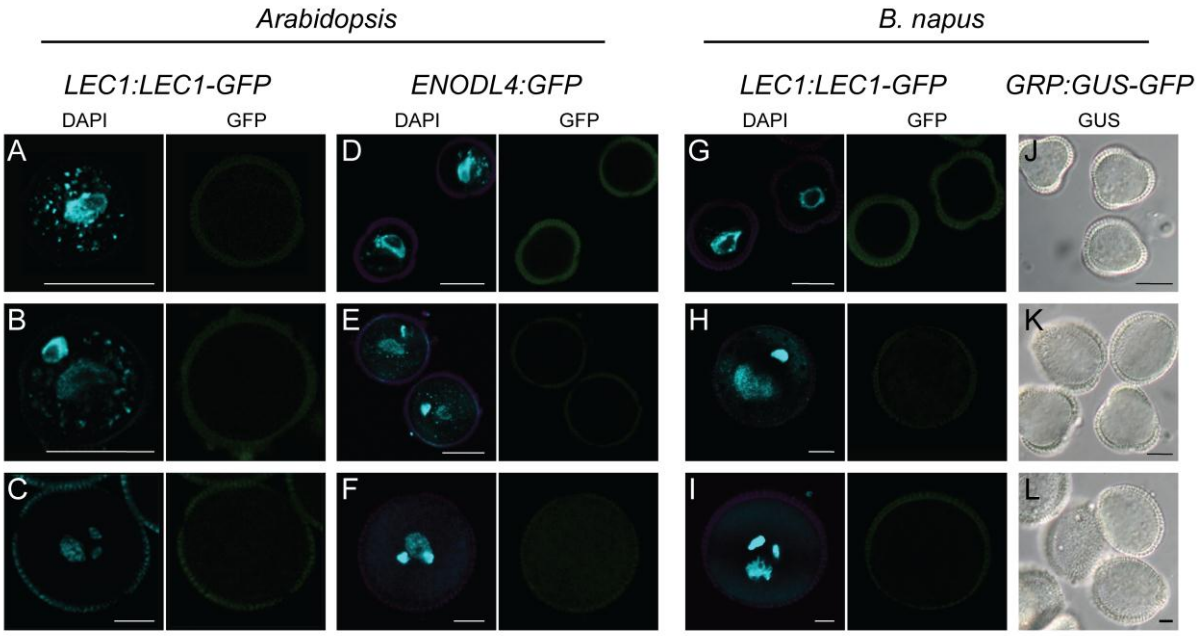
**(G)** Transition stage embryo.

**(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.

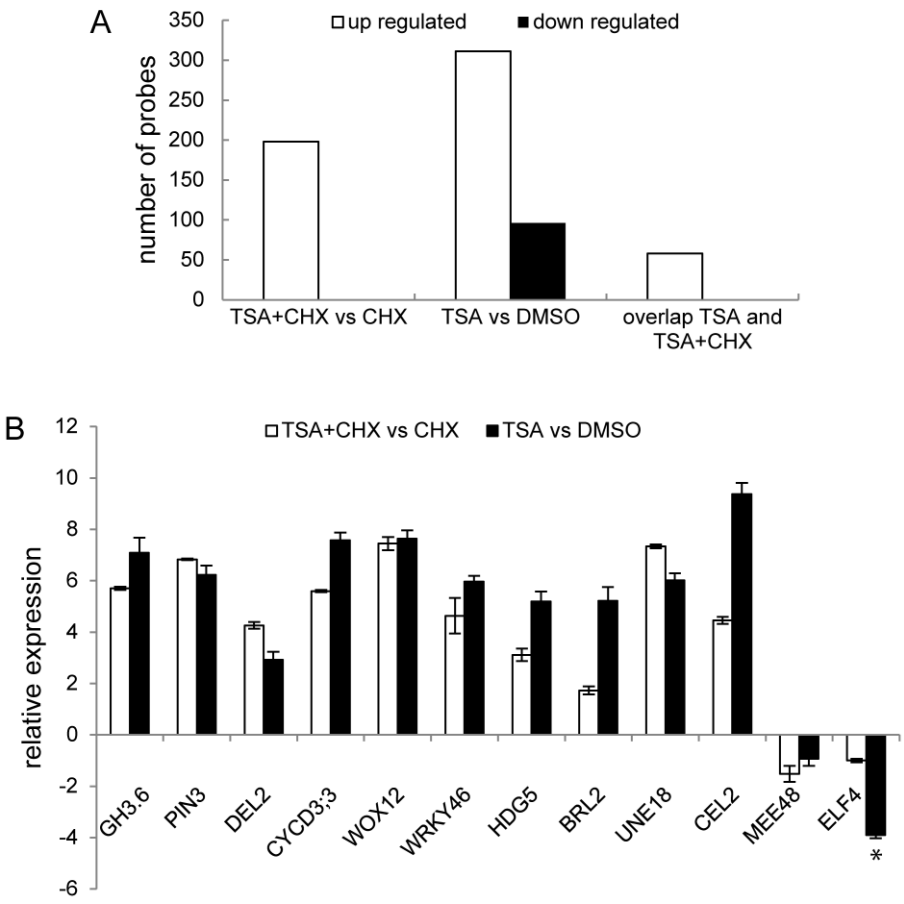
**(D-F)** *ENODL4:GFP* expression in Arabidopsis.

**(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.

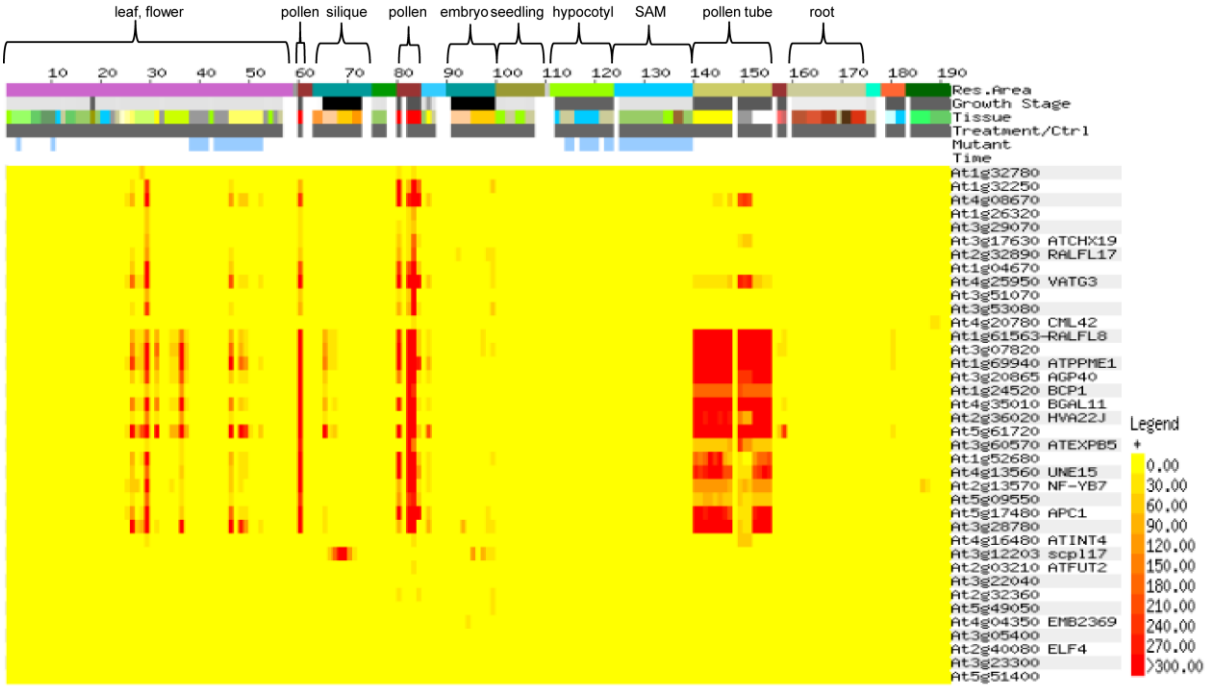
Uninucleate (upper), binucleate (middle) and trinucleate (lower) stages of pollen development. Scale bar, 10 µm.




**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).

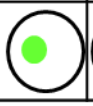
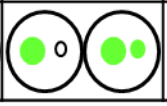
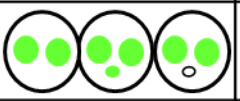



**Supplemental Figure 9.** TSA-down regulated genes are preferentially-expressed in pollen and pollen tubes.

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.

**Supplemental Table 1.** Summary of the expression patterns of the *LEC1:LEC1-GFP* and *GRP:GFP-GUS* reporters in *B. napus* microspore culture.

GFP expression in the nuclei or cells is indicated in green. The proportion of GFP positive cells in each sample is shown in Supplemental Table 1.

| | | 2 days | | | | 5-8 days | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | micro spore | Binucleate pollen | Trinuclea te pollen | Sprophytic division | Type I | Type II | Type III | Type IV |
| *LEC1: LEC1-GFP* | control |  |  |  |  | + | + | + | + |
| | TSA |  |  |  |  | + | + | + | + |
| *GRP: GFP-GUS* | control | | | | | + | + | - | + |
| | TSA | | | | | + | + | - | + |

**Supplemental Table 2**. DNA Primers used for genotyping *hda* T-DNA insertion lines.

| Gene | Gene ID | ABRC stock number | Gene-specific forward primer (5'-3') | Gene-specific reverse primer (5'-3') |
|---|---|---|---|---|
| *HDA2* | At5g26040 | SALK_041074C | CATTTATGGATTGGCATTTCG | GGAACTTGCAAACTCTACCCC |
| *HDA5* | At5g61060 | SALK_024514 | AGAGGATAGGTCAAGAAGCCG | CGTCAATACATAGCAGGCCA |
| *HDA6* | At5g63110 | FLAG_599A10 | TTTGTACCTCACGGTGGAAAG | CAAAAAGTCCGTCGAAGACAG |
| *HDA7* | At5g35600 | SALK_002912C | TTATCGACTTGATCCTCTCGG | TATGGCAATATCGGCTTCTTG |
| *HDA8* | At1g08460 | GK-100A06 | AGAAAGCCAAAATCCAGAACC | GAATTCAATGCCAGCTTGACAG |
| *HDA9* | At3g44680 | SALK_007123 | TTCTTGTTGATGATTGGAGCC | TTGAAACCGTCCTCACAAATC |
| *HDA10* | At3g44660 | SALK_119279 | GTCCAGCAAAGACTCACAAGC | CGTTTTCAGGAATCTCTGCAG |
| *HDA14* | At4g33470 | SALK_097006 | TCACGCAAAAATGTTAGAGGC | AGGGAAGTTTGGTTAACCGTG |
| *HDA15* | At3g18520 | SALK_004027C | CTTCTCTGTTCATCGTTTCGC | AGCAACATTCTCTCGTCGAAC |
| *HDA17* | At3g44490 | SALK_090088 | GTTGCAGTCTTTGGAGCTCAG | AGCTCAATTGAGCAGTGTTGG |
| *HDA18* | At5g61070 | SAIL_1289_F05 | TGAAGCAATCTTGTTTCTCCG | TTCCCTCCAGATTCAGTGTTG |
| *HDA19* | At4g38130 | SALK_139445 | ACTCTCTTCCTTGTCTGCGTG | ACCAGACAATGAATCAGCACC |
| *HDT1* | At3g44750 | GK-355H03-016870 | CGGCTTCGTATTAAAACCCTC | GCCTTTGGTTTAGCTACAGCC |
| *HDT2* | At5g22650 | SAIL_1247_A02 | TCTTCTTCTCCTCCCGATAGC | CGTTGGCAGTAACAGCAGTAG |
| *HDT3* | At5g03740 | SALK_002860.41.15.x | CATGATTCATGCCGAGAACTC | CTTCAACACCTGCAAACGCA |
| *HDT4* | At2g27840 | GABI_836B08 | GTGATAACGACATTGCCCAAC | CTGAGAGGCATGGACCATG |
| T-DNA border/collection | | | | primer |
| Left border/GABI-KAT T-DNA insertion lines | | | | ATATTGACCATCATACTCATTGC |
| Left border/SALK T-DNA insertion lines | | | | ATTTTGCCGATTTCGGAAC |
| Left border/SAIL T-DNA insertion lines | | | | GAAATGGATAAATAGCCTTGCTTCC |
| Left border/FLAG T-DNA insertion lines | | | | CGTGTGCCAGGTGCCCACGGAATAGT |

**Supplemental Table 3**. DNA primers used for RT-PCR and microarray validation.

| RT-PCR | | | |
|---|---|---|---|
| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | |
| *Actin* | AAGAGCAGTTCTTCGGTGGA | CAATCCCCATCTCAACAAGG | |
| *FUS3* | TACAACAGCGACAACGGAAA | TTTTCATATCGGGCATGGAT | |
| *LEC1* | AAACGGCAGAGAAACAATGG | TGAAACCCCATACCACTTCC | |
| *LEC2* | GATGTTGACTCTGTGCAGTCTTG | TTGGGAACAAACACATTTTATCA | |
| *BBM1* | GGAAGATGGCAAGCTAGGATAG | TCACTAGCAGCGTAGGCATC | |
| *BNM1* | GGCAACGTTCTCAGTTCTGTC | GACGGTGGATGTTCTCATCA | |
| Microarray validation (quantitative real time RT-PCR) | | | |
| *Gene* | probe ID | Forward primer (5'-3') | Reverse primer (5'-3') |
| *GH3.6* | rres060198.v1 | GAACAGGCTCCAGTTCATCG | AGTCTCACGGTCGGTTCGTC |
| *MEE48* | rres088597.v1 | CGAGGACGCATCATCTCCAA | CTGTGCGGATGTTGGAGCCA |
| *WOX12* | rres120947.v1 | CTACTCGGCCTCTCTTCTTC | GGATGCTGGAGTTCTGATCAG |
| *PIN3* | rres130992.v1 | GACGCTGAGAATGGACTGAAC | GCGGCATATGTTTCCCTTGAC |
| *DEL2/E2FD* | rres085986.v1 | GCGACTGTATAACCGAGACG | GCTACAAGCCCAATGCTCTC |
| *WRKY46* | rres114866.v1 | GGAGAGAGCTTGCCAAACG | CTTCGATCTCGCGGCTTCTC |
| *ELF4* | rres038370.v1 | CAATACCAAATTCTCATCGGCG | CATTGGTTAACCGACTGGTTC |
| *CYCD3;3* | rres059022.v1 | GTGCATGAGCAGCAGATGAAG | CTTACCATAGCCGCTAATATGC |
| *HDG5* | rres059323.v1 | GTCCGGGACTGAGTTTGATG | GGATCTGACGATCAGTATGTC |
| *BRL2* | rres142640.v1 | GAAGGTTCGGAGACTCAAGAAG | CCACTTGTAACATATTAGGCCG |
| *UNE18* | rres071830.v1 | CTGAACAGGTCAGCCATCAC | GGTAGAAGTCGATTGAATTGCC |
| *CEL2* | rres115173.v1 | CAATGCCACGATGGCTTCTC | CTGGTAGTCTGACCGTTCATC |
| *SAND* | At2G28390 | AACTCTATGCAGCATTTGATCCACT | TGATTGCATATCTTTATCGCCATC |

**Supplemental Dataset 1.** Developmental pathways found in control and TSA-treated cultures.

**Supplemental Dataset 2.** Significantly differentially-regulated genes observed by microarray analysis.

## Supplemental References:

**Livak, K.J., and Schmittgen, T.D.** (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. Methods **25:** 402-408.

**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.