Changes in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley

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Abstract

The microspore can be induced *in vitro*, under specific stress treatments, to deviate from its gametophytic development and to reprogram towards embryogenesis, becoming a totipotent cell and forming haploid embryos which can further regenerate homozygous plants for production of new isogenic lines, an important biotechnological tool for crop breeding. DNA methylation constitutes a prominent epigenetic modification of the chromatin fibre which regulates gene expression. Changes in DNA methylation accompany the reorganization of the nuclear architecture during plant cell differentiation and proliferation, however, global DNA methylation and genome-wide expression patterns relationship is still poorly understood.

In this work, the dynamics of global DNA methylation levels and distribution patterns have been analyzed during microspore reprogramming to embryogenesis and during pollen development in *Hordeum vulgare*. Quantification of global DNA methylation levels and 5-methyl-deoxy-cytidine (5mdC) immunofluorescence has been conducted at specific stages of pollen development and after reprogramming to embryogenesis, to analyze the epigenetic changes that accompany the change of developmental programme and cell fate.

Results showed low DNA methylation levels in microspores and a high increase along pollen development and maturation; an intense 5mdC signal was concentrated in the generative and sperm nuclei whereas the vegetative nucleus exhibited lower DNA methylation signal. After the inductive stress treatment, low methylation levels and faint 5mdC signal were observed on nuclei of reprogrammed microspores and 2-4 cell proembryos. This data revealed a global DNA hypomethylation during the change of the developmental programme and first embryogenic divisions, in contrast with the hypermethylation of generative and sperm cells of the male germline accomplished during pollen maturation, suggesting an epigenetic regulation after microspore embryogenesis induction. At later embryogenesis stages global DNA methylation progressively increased, accompanying embryo development and differentiation events, like in zygotic embryos, supporting that DNA methylation is critical for the regulation of microspore embryogenesis gene expression.

Introduction

After specific stress treatments, the *in vitro*-cultured microspore at the vacuolated developmental stage (González-Melendi et al. 1995), can reprogram and initiate an embryogenesis program producing multicellular embryos. Homozygous plants can further regenerate from the embryos for producing new isogenic lines, which are important biotechnological tools for crop breeding. The microspore, after the external stress signals, becomes a totipotent cell because it can develop into an embryo and subsequently an entire plant, therefore acquiring the potential and embryogenic competence to give rise to all the cell types, tissues and organs that make up the plant body (Verdeil et al. 2007, Grafi et al. 2011). In barley, microspore embryogenesis has been induced in microspore *in vitro* cultures by cold and starvation stress treatments (Kasha et al. 2001; Coronado et al. 2005; González-Melendi et al. 2005, Rodríguez-Serrano et al. 2012).

Isolated microspore cultures constitute very convenient systems to analyze the subcellular mechanisms underlying cell reprogramming, totipotency acquisition and subsequent embryo formation. In recent years, increasing amounts of information have reported the presence of genes and molecules controlling early embryogenic events, but knowledge on the genetic control of the process and the possible involvement of external factors regulating embryo growth and development is still scarce (El-Tantawy et al. 2013). Little is known about the mechanisms that induce the dedifferentiation of a single somatic cell into a totipotent embryogenic cell. Several reports have related totipotency of cells with an open chromatin conformation characterized by large nuclei and homogenous euchromatin (Grafi et al. 2011). There are increasing evidences that numerous processes of development and differentiation in both plants and animals are accompanied of chromatin remodeling (Kouzarides 2007). Stress-induced plant cell reprogramming involves changes in global genome organization, being the epigenetic modifications key factors of genome flexibility (Arnold-Schmitz 2004, Solís et al. 2012).

DNA methylation constitutes a prominent epigenetic modification of the chromatin fiber which is locked in a transcriptionally inactive conformation leading to gene silencing (Kohler et al. 2012). Generally, open chromatin increases the accessibility of

the genome to transcription machinery, while closed chromatin represses gene expression by limiting the accessibility (Reyes 2006, Kouzarides 2007). The past decade revealed exciting findings on epigenetic mechanisms controlling developmental processes specific to flowering plants: the determination of sporogenic fate during development, the differentiation of gametes within multicellular gametophytes, and the distinction of the two male gametes involved in double fertilization (Twell 2011). Recent studies have demonstrated epigenetic changes during plant developmental processes and after microspore induction to the sporophytic pathway conducting to embryogenesis (Solís et al. 2012, Testillano et al. 2013). However, the knowledge of the DNA methylation regulation during microspore embryogenesis is very limited.

In this work, the dynamics of global DNA methylation levels and distribution patterns was analyzed during microspore reprogramming to embryogenesis in comparison with pollen development in *Hordeum vulgare*. Our results revealed a global DNA hypomethylation during the change of developmental program and the first embryogenic divisions, in contrast with the hypermethylation of the generative and sperm cells of the male germline accomplished during pollen maturation, as well as in the embryo cells during embryo differentiation. These results suggest an epigenetic regulation after microspore embryogenesis induction and subsequent embryo development supporting the idea that DNA methylation is critical for the regulation of microspore embryogenesis gene expression.

Materials and Methods

Plant material and growth conditions

Winter barley cultivars, *Hordeum vulgare* L. cv. Igri were used as donor plants. Seeds were germinated in soil for 1 month at 4ºC. After that, they were grown at 12ºC with a 12/12 light/dark cycle (10,000–16,000 lx) for 1 month in a plant growth chamber (Sanyo) (relative humidity about 70%), and then in a greenhouse under a controlled temperature of 18ºC.

Microspore isolation and culture

Spikes containing microspores at the vacuolated stage were collected and surface sterilized by immersion in bleach at 5% for 20 min, followed by 3–4 washes with sterile distilled water. The sterilized spikes were then pre-treated at 4ºC for 23–24 days as stress treatment to induce embryogenic development. The isolation and culture of the microspores were performed as previously described (Rodríguez-Serrano et al. 2012) with final density of 1.1 x 10^5 cell per mL in an appropriate volume of KBP medium (Kumlehn et al. 2006).

Processing for microscopy analysis

Samples from different culture times were collected and fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.3, washed in PBS, dehydrated in an acetone series, embedded in Historesin Plus at 4º C and sectioned at 2 μm thickness using an ultramicrotome (Ultracut E Reichert). Semithin sections were collected on slides, stained with toluidine blue and observed under bright field microscopy.

Immunofluorescence

Immunolocalization of 5-methyl-deoxy-cytidine (5mdC) was performed as previously described (Solís et al. 2012, Testillano et al. 2013). Historesin semithin sections were mounted on 3-aminopropyltriethoxysilane- coated slides, denatured with 2 N HCl for 45 min, washed in PBS and treated with 5% bovine serum albumin (BSA) in PBS for 10 min, incubated with anti-5mdC mouse antibody (Eurogentec) diluted 1/50 in 1% BSA and Alexa-Fluor-488 anti-mouse IgG antibody (Molecular Probes) diluted 1/25.

Sections were counterstained with 1 mg mL^{-1} DAPI (4',6-diamidino-2-phenylindole) for 10 min and analyzed by confocal microscopy (TCS-SP5, Leica). As negative controls, either DNA denaturation or the first antibody was omitted. Also, as negative control, immunodepletion was carried out by preblocking the antibody with 5mdC at 4°C overnight prior to immunofluorescence as described (Testillano et al. 2013).

Quantification of global DNA methylation

Genomic DNA was extracted from microspores and mature pollen directly isolated from anthers, and from different microspore culture stages using a plant genomic DNA extraction kit (DNeasy Plant Mini, Qiagen) as described (Solís et al. 2013). A MethylFlash Methylated DNA Quantification Kit (Colorimetric) (Epigentek, NY) was used according to the manufacturer's instruction using 200 ng of genomic DNA for each sample (Li and Liu 2011, Testillano et al. 2013) for the quantification of the DNA methylation. Three biological and two analytical replicates per sample were taken. P-values were calculated using Student's t -test.

Results

During the gametophytic development in vivo, the haploid microspore undergoes a long interphase with the formation of a large vacuole which pushes the nucleus to the periphery forming the so-called vacuolated microspore (Fig. 1A). At this stage, the first asymmetric mitosis occurs originating the bicellular pollen (Fig. 1B) with two very different cells: the small generative cell immersed in the cytoplasm of the large vegetative cell. The generative nucleus ("Gn" in Fig. 1B) exhibits a highly condensed chromatin, while the vegetative nucleus ("Vn" in Fig. 1B) shows a less condensed chromatin and a large nucleolus. In barley, the second pollen mitosis occurs before anthesis forming the tricellular pollen grain (Fig. 1C) which contains the two small sperm cells (arrows in Fig. 1C) inside the vegetative cell. With the progression of pollen maturation the vacuole disappears and the cytoplasm is occupied by storage products and small vacuoles (Fig. 1C).

To induce the microspore embryogenesis process in barley, a cold stress treatment was applied to the vacuolated microspores which changed the gametophytic pathway to an embryogenic development (Rodríguez-Serrano et al. 2012). After the stress treatment, responsive microspores reprogrammed and divided symmetrically originating two-cell structures (Fig. 1D) with two nuclei of similar size and structural organization. After 4-6 days in culture, further divisions gave rise to multicellular embryos, still surrounded by the microspore wall, the exine (Fig. 1E) which later broke permitting a faster proliferation and the formation of larger multicellular embryos (Fig. 1F) at 9-10 days. As embryogenesis proceeded, more developed embryos were observed in 15 days of culture (Fig. 1G), and completely formed mature embryos were formed by 30 days (Fig. 1H, inset).

For the analysis of the changes in genomic DNA methylation during pollen development and microspore embryogenesis, the quantification of the percentage of methylated DNA was performed in selected stages of the two developmental pathways, the gametophytic and the embryogenic programs. Results revealed a very low proportion of methylated DNA in vacuolated microspores and a high increase with the progression of the pollen development and maturation, reaching a much higher

proportion in mature pollen (Fig. 2A). On the contrary, samples after the inductive stress containing reprogrammed microspores and 2-4 cell embryos showed low levels of DNA methylation (Fig. 2B). At later embryogenesis stages, DNA methylation levels progressively increased in developing embryos of 15 days, and mature embryos of 30 days (Fig. 2B). The global DNA methylation percentage of mature pollen was the highest, seven-fold more than vacuolated microspores (Fig. 2A), and near two-fold more than mature embryos (Fig. 2B).

Confocal microscopy analysis of the 5-methyl-deoxy-cytidine (5mdC) immunofluorescence assays revealed the nuclear distribution of methylated DNA and showed differences in the intensity and distribution pattern of the immunofluorescence signals at defined developmental stages. Confocal analysis were performed using the same laser excitation and sample emission capture settings for image acquisition in all immunofluorescence preparations, this procedure permitted an accurate and reliable comparison between signals from cells at different developmental stages.

During the gametophytic development, the vacuolated microspore showed a faint fluorescence 5mdC signal distributed as very small spots over chromatin regions (Fig. 3A-D), the large central nucleolus appeared negative for 5mdC immunolocalization and for DAPI staining (Fig. 3B-D). After the first mitosis, the two nuclei of the bicellular pollen showed different 5mdC distribution patterns (Fig. 3E-H), the generative nucleus exhibited higher immunofluorescence signal than the vegetative one and the signal formed a thick reticulum covering the entire generative nucleus, whereas the vegetative nucleus showed very few or no signals (Fig. 3G, H). Finally, in mature tricellular pollen, 5mdC fluorescence was very intense on the two sperm nuclei, covering almost the whole nuclear areas; the vegetative nucleus, with a lobulated shape at this late developmental stage, showed 5mdC labeling as small spots on a thin reticulum throughout the nucleus (Fig. 3I-L).

The labeling pattern of 5mdC was different after the stress treatment for embryogenesis induction than in developing pollen grains. In samples collected just after the stress, some microspores still appeared uninucleated (Fig. 4A-D), while other microspores had already divided producing 2-4 cell structures or proembryos (Fig. 4E-H). Nuclei of the reprogrammed microspores and small proembryos showed low 5mdC fluorescence,

with a diffuse signal (Fig. 4B-D) or forming a very thin reticulum (Fig. 4F-H) over the DAPI-stained nuclei. At later embryogenesis stages, the multicellular embryos surrounded by the exine (Fig. 4I-L) and after the exine breakdown (Fig. 5A-C), were formed at 5-10 days after the stress treatment and exhibited large rounded nuclei. The cell nuclei of multicellular embryos showed different labeling intensities (Fig. 5A-C), probably corresponding to nuclei at different interphase periods of the cell cycle which presented different chromatin condensation states; the nucleoli did not show fluorescence in any case, appearing as dark rounded regions in both DAPI and 5mdC micrographs (Figs. 4I-K, 5A-C). A few interphasic nuclei did not show labeling (arrowheads in Fig. 5B) whereas other nuclei appeared with medium fluorescence intensity. Mitotic nuclei were observed in multicellular embryos and exhibited high fluorescence which covered the condensing chromosomes of prophasic nuclei (arrow in Fig. 5B) and chromosomes at different mitotic phases. With the progression of embryo development, larger embryos were formed and displayed a heterogeneous pattern of 5mdC labeling in the nuclei (Fig. 5D-F), most embryo cell nuclei showed fluorescence of different medium-high intensity and some nuclei did not show any detectable signal (Fig. 5D-F).

Immunofluorescence control experiments performed by eliminating either the denaturation treatment or the first antibody as well as immunodepletion experiments completely abolished the signal in all samples (figures not shown).

Discussion

The results reported in this study illustrate for the first time the epigenetic changes, regarding DNA methylation, during pollen development and after embryogenesis induction in barley microspore cultures. The chromatin remodeling occurring during development of male gametes provide generative and sperm nuclei with a unique chromatin organization in a highly condensed state. The high increase of global methylation observed in the present work during pollen maturation in barley was associated with the heterochromatization that accompanied cellular differentiation in the most advanced stages of pollen development. In plants, the male germline is represented by the generative cell and the two sperm cells. Some reports have indicated the specific accumulation of epigenetic histone variants in the nuclei of the male germline (Ingouff et al. 2007), as well as a variable epialleles hypermethylation in the male germline (Borges et al. 2012). The results presented here in barley revealed a differential genome-wide hypermethylation in the generative and sperm nuclei, while the vegetative nucleus remained hypomethylated after the first pollen mitosis.

The distribution of several histone modifications has been recently reported in the generative, sperm and vegetative nucleus of barley pollen (Pandey et al. 2013); in this report, marks associated with transcriptional activity, like active RNA Polymerase II, acetylated histone H3 in lysine 9, H3K9Ac, and tri-methylated histone H3 in lysine 27, H3K27me3 (Liu et al. 2010) appeared very low or absent in generative and sperm nuclei, whereas late microspore and vegetative nuclei exhibited higher labelling (Pandey et al. 2013). Our results on DNA methylation in pollen nuclei are in agreement with these findings and provide new evidences that gene silencing-related epigenetic modifications are enriched in the generative and sperm nuclei while activity-related marks are mainly localized in late microspore and vegetative nuclei. The repressive epigenetic mark di-methylated histone H3 in lysine 9, H3K9me2, has been found not only in the generative and sperm nuclei but also in the vegetative and microspore nuclei of barley pollen, and the activating mark di-methylated histone H3 in lysine 4, H3K4me2, has been also localized in microspore and all pollen grain nuclei (Pandey et al. 2013), the authors suggested that these modifications would be related to local

changes in transcriptional activity and tissue-specific regulated genes and would be therefore present in nuclei of all stages of pollen development.

Low DNA methylation levels have been reported in microspores and vegetative nuclei of *Brassica napus*, as well as a punctuate 5mdC distribution nuclear pattern (Solis et al, 2012) corresponding to the scarce heterochromatin masses typical of this species (Seguí-Simarro et al 2011). In barley, the results revealed low DNA methylation and punctuate-thin reticulum distribution patterns of 5mdC in microspores and vegetative nuclei, in relation to the low condensed chromatin pattern of this monocot plant and with the high transcriptional activity reported for the vacuolated microspore (Testillano et al. 2000, 2005), the most responsive developmental stage for embryogenesis induction in many species (González-Melendi et al 1995, Maluzinsky et al. 2003, Bárány et al. 2005, Prem et al. 2012).

Immunofluorescence assays showed no 5mdC signal over the nucleolus of vacuolated microspores. In the nucleolus, rRNA genes are transcribed, being the nucleolar architecture a clear reflect of the level of ribosome biosynthesis activity. In many organisms including plants, rDNA occurs in high copy numbers of genes which can be silent or transcribed depending on the activity state of the cell. Epigenetic changes involving DNA methylation and histone modifications have been reported to act in the dosage regulation of the number of active rRNA genes at any one time (Lawrence et al. 2004, Galetzka et al. 2006, Preuss et al. 2008, Bartova et al. 2010). In contrast with animal cells, plant cells do not usually exhibit a shell of perinucleolar heterochromatin where the silent portion of the repeated ribosomal genes and some telomeric and centromeric DNA regions were found (Bartova et al. 2010, Politz et al. 2013), only a very few condensed chromatin knobs containing inactive rDNA can be found at the nucleolar periphery (Testillano et al. 2005) and could correspond with some of the 5mdC spots found at the periphery of the nucleolus in the vacuolated microspore (Fig. 3C). In plants, low active nucleolus displayed small condensed chromatin masses, containing silent rDNA, at the nucleolar interior in the so-called heterogeneous fibrillar centers, whereas in very active nucleolus rDNA is distributed in a decondensed state throughout the nucleolar dense fibrillar component (Risueño and Testillano 1994, Testillano et al. 2005). The large nucleolus of the vacuolated microspore has been characterized with a typical organization of high transcriptional activity, corresponding

to the G2 phase of the cell cycle (Risueño and Testillano 1994, González-Melendi et al. 1995, Testillano et al. 2005), the ribosomal chromatin being distributed in a decondensed state through the fibrillar component of the nucleolus, as revealed by rDNA in situ hybridization (Risueño and Testillano 1994, Testillano et al. 2005). In barley microspores, the nucleolus did not show 5mdC labeling indicating a decondensed state of ribosomal chromatin which correlates with an active rDNA transcription at this developmental stage.

In contrast with the microspore and vegetative nuclei, generative and sperm nuclei 5mdC signals were intense and distributed in wider nuclear regions covering the large heterochromatin masses that occupied the major part of the nuclear volume. In *Brassica napus*, hypermethylation of mature pollen was correlated with up-regulation of *BnMET1* methyl transferase, suggesting the involvement of MET1 in the methylation of generative nuclei (Solís et al. 2012), but no data is available in barley on the MET1 participation on epigenetic mechanisms during pollen development. The results obtained indicated an important change in global DNA methylation specifically in the male germline of barley, process probably contributing to the epigenetic inheritance after fertilization that has been reported in many plant species (Calarco et al. 2012).

Cell reprogramming by stress involves morphological and physiological changes as well as modifications in the genome organization and activity, as reported in dicot plants (Arnholdt-Schmitt, 2004; Miguel and Marum, 2011, Solis et al. 2012), the present study shows in a monocot species, that in contrast with the DNA methylation increase in the gametophytic development, microspore reprogramming to embryogenesis was associated with very low levels of global DNA methylation. Significant variations in global DNA methylation have been related to global changes of gene expression occurring during plant vegetative developmental processes (Meijón et al., 2010). The present data reveal an epigenetic change associated with the microspore reprogramming to a new developmental program and the first embryogenic divisions, epigenetic change that can be related to a global change of gene expression reported by transcriptomic analysis (Maraschin et al., 2006). Thus, our results indicate, for the first time in a monocot species, the existence of epigenetic changes after pollen embryogenesis induction that could be associated with the acquisition of embryogenic

competence by the microspore and the ability to erase its gametophytic program and switch to a new cell fate.

At later stages of microspore embryogenesis, the results of the present study show a mild increase in global DNA methylation levels in multicellular embryos whose cells showed 5mdC localization patterns covering the nuclear volume with 5mdC signals of different intensity and distribution. It has been recently reported that nuclei of cycling cells of root meristems exhibited different signal intensities and distribution patterns of 5mdC immunofluorescence related to different interphase periods of the cell cycle presenting different chromatin condensation states (Testillano et al. 2013). Most cells of the early microspore-derived multicellular embryos were in active proliferation, as revealed in several monocot and dicot species (Testillano et al 2002, 2005, Bárány et al. 2005); in the present study, nuclei of young barley multicellular embryos presented distribution patterns of 5mdC which varied in intensity and localization, similar to the patterns related to the different chromatin condensation states observed in plant cycling cells.

The quantification of DNA methylation performed during advanced stages of microspore-derived embryo development revealed a gradual DNA methylation increase. Recent reports have shown an increase in global DNA methylation during the progression of in vitro somatic embryogenesis of the pineapple *Acca sellowiana* (Fraga et al. 2012), as well as during microspore embryogenesis of rapeseed (Solís et al. 2012). Since the progression of embryogenesis is accompanied by cell differentiation events, the results presented would indicate that the increase of global DNA methylation levels in microspore-derived embryos is related to the cellular differentiation, as found in other plant systems (Costa and Shaw, 2007, Solís et al. 2012). Differences in the distribution pattern of 5mdC between proliferating and differentiating plant cells have been established in various plant systems (Testillano et al. 2013, Solis et al. 2012), cells in differentiation showing much higher 5mdC labeling. The different 5mdC localization patterns found in cells of the advanced developing barley embryos could reflect different chromatin states of dividing cells and differentiating embryo cells.

During the zygotic embryogenesis, hypomethylation in the endosperm is accompanied by an extensive hypermethylation in the embryo (Köhler et al. 2012). This process has

been suggested to ensure silencing of transposons and repetitive elements in the embryo and it occurs in monocots as well as in dicots, implicating an evolutionarily conserved mechanism of DNA hypermethylation in the embryo (Köhler et al. 2012). The results of the present work also revealed a hypermethylation process during microspore-derived embryo development in barley providing new evidences of analogous mechanisms acting in microspore embryogenesis and zygotic embryogenesis and supporting that DNA methylation is critical for the regulation of plant embryogenesis gene expression.

Taken together, the results presented revealed epigenetic changes that accompany the two pollen developmental programs analyzed: pollen maturation and microspore embryogenesis, in barley, suggesting the possible involvement of DNA methylation dynamics in regulating microspore embryogenesis induction and progression in a monocot species.

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Figure legends

Figure 1: Main stages of pollen development and microspore embryogenesis in barley. Semithin sections, toluidine blue staining. (A-C) Male gametophytic development *in vivo*. (D-H) Microspore embryogenesis *in vitro*. (A) Vacuolated microspore. (B) Young bicellular pollen with the vegetative and generative cells. (C) Mature tricellular pollen with the vegetative nucleus and the two small sperm cells (arrows). (D) Two-cell structure after microspore embryogenesis induction. (E) Multicellular embryo still surrounded by the pollen wall, the exine. (F) Multicellular embryo just after the exine breakdown. (G) Developing embryo after 15 days in culture. (H) Mature embryo formed after 30 days in culture, region showing the peripheral cell layer of the protodermis, inset: panoramic view of several embryos in culture. Ex: exine, V: vacuole, Vn: vegetative nucleus, Gn: generative nucleus. Bars: A-F, 20 µm; G-H: 100 µm.

Figure 2: Quantification of global DNA methylation during pollen development and microspore embryogenesis. Histograms representing the mean values of 5mdC percentage of total DNA in different developmental stages of pollen gametophytic development (A) and microspore embryogenesis (B). Each column represents an average of three independent biological and two technical replicates per sample. Bars with different letters indicate developmental stages in which the mean percentage values are significantly different at $P < 0.001$.

Figure 3: 5mdC immunolocalization during pollen development. A-D: Vacuolated microspore, E-H: Bicellular pollen, I-L: Mature tricellular pollen. A, E, I: Differential interference contrast (DIC) images of the cell structure. B, F, J: DAPI staining of nuclei (blue). C, G, K: 5mdC immunofluorescence (green), higher magnification. D, H, L: Merged images of DAPI (blue) and 5mdC immunofluorescence (green). The same structures are visualized under different microscopy modes from A to D, from E to H and from I to L. The exine showed unspecific autofluorescence under UV excitation in DAPI images. Vn: vegetative nucleus, Gn: generative nucleus, Arrows: sperm cells. Bars: A, B, E, F, I, J, 10 µm; C, D, G, H, K, L, 20 µm.

Figure 4: 5mdC immunolocalization during early stages of microspore embryogenesis. A-H: Vacuolated microspores and two-cell structures after embryogenesis induction. I-L: Multicellular embryo still surrounded by the exine. A, E, I: DAPI staining of nuclei (blue). B, F, J: 5mdC immunofluorescence (green). C, G, K: Merged images obtained by differential interference contrast (DIC) to show the cell structure, DAPI (blue), and 5mdC immunofluorescence (green). D, H, L: Higher magnification of DAPI (blue) and 5mdC (green) merged images. The same structures are visualized under different microscopy modes from A to D, from E to H and from I to L. The exine showed unspecific autofluorescence under UV excitation in DAPI images. Bars: A-C, E-G, I-K, 20 µm; D, H, L, 10 µm.

Figure 5: 5mdC immunolocalization during advanced stages of microspore embryogenesis. A-C: Multicellular embryo just after the exine breakdown. D-F: Developing embryo after 15 days in culture. A, D: 5mdC immunofluorescence (green). B, E: Merged images of DAPI (blue) and 5mdC immunofluorescence (green). C, F: Merged images obtained by differential interference contrast (DIC) to show the cell structure, DAPI (blue) and 5mdC (green) fluorescence signals. Arrows in A and B point mitotic chromosomes. Arrowheads in B and E point nuclei which did not show 5mdC labelling. Arrowheads in C point exine remnants still attached to the periphery of the multicellular embryo. The same structures are visualized under different microscopy modes from A to C, and from D to F. Bars: A-C, 25 µm; D-F, 75 µm.

Figure 1

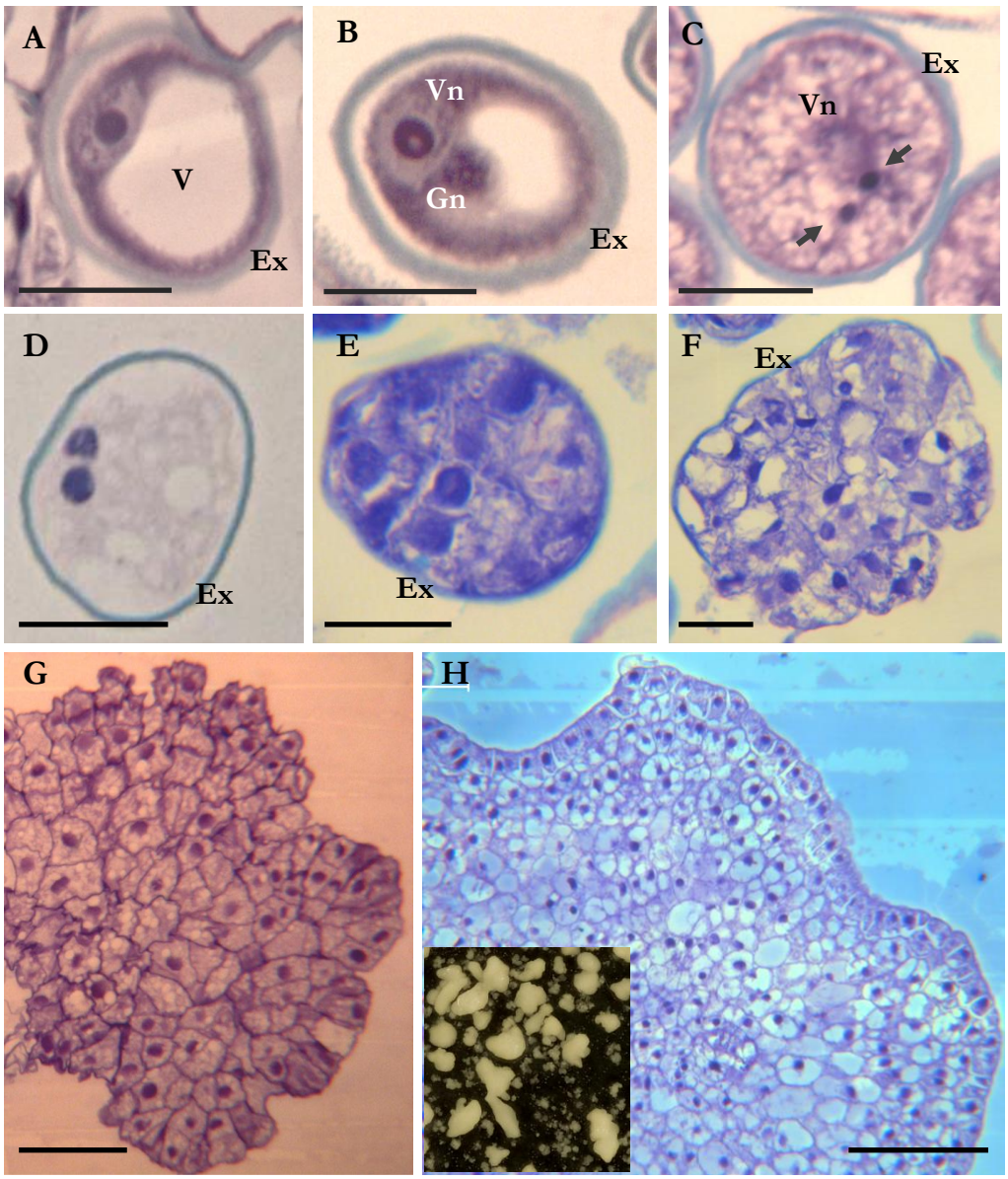


Figure 2

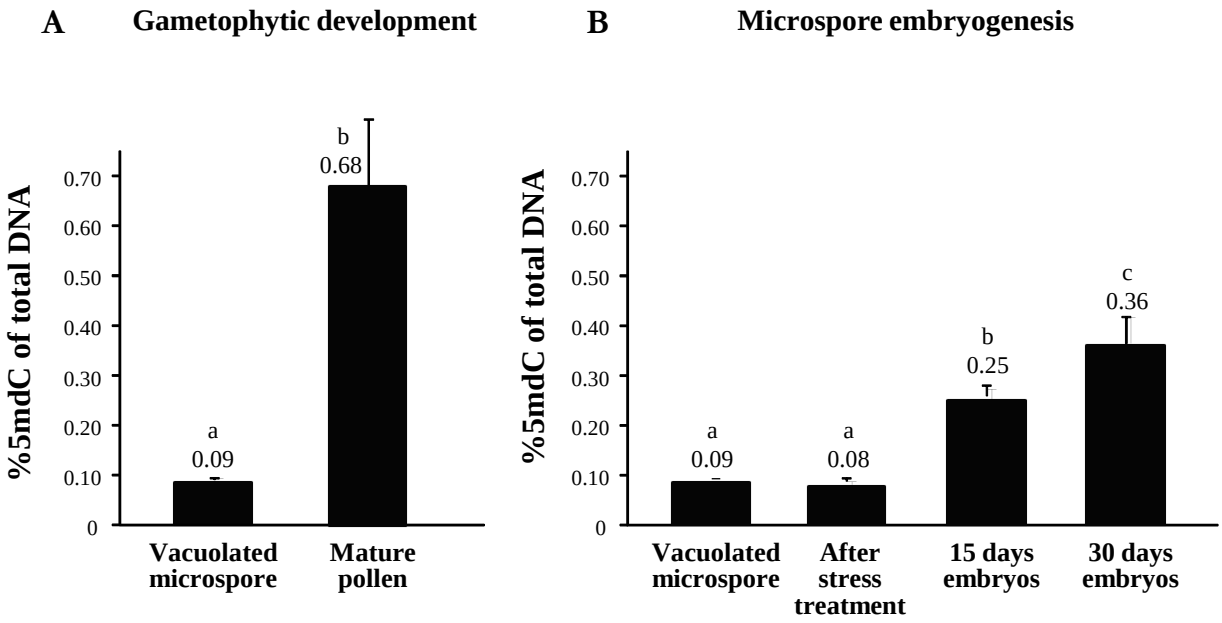
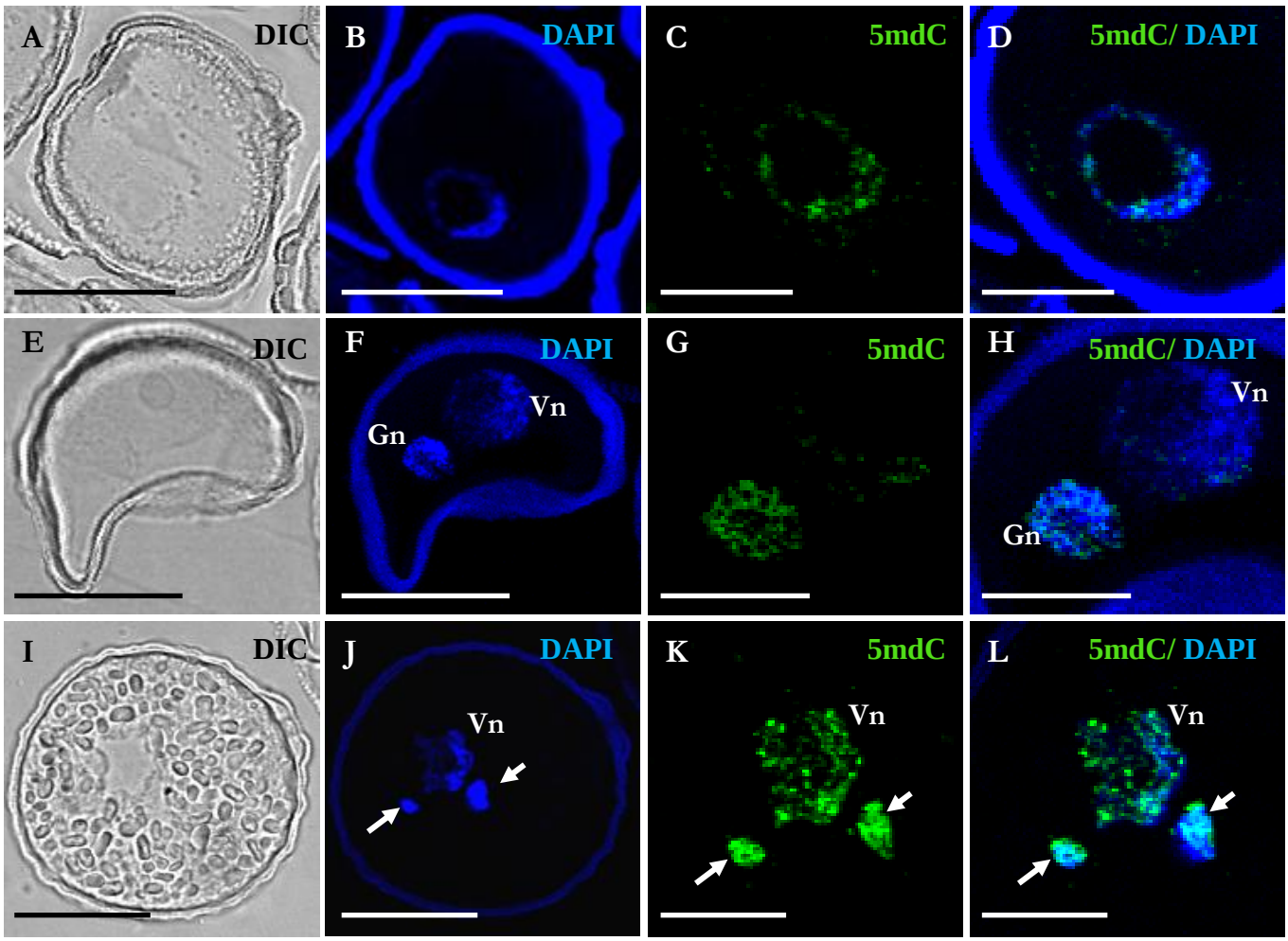

A
Gametophytic development
%5mdC of total DNA
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
a
0.09
b
0.68
Vacuolated microspore
Mature pollen
B
Microspore embryogenesis
%5mdC of total DNA
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
a
0.09
a
0.08
b
0.25
c
0.36
Vacuolated microspore
After stress treatment
15 days embryos
30 days embryos

Figure 3


A
DIC
B
DAPI
C
5mdC
D
5mdC/ DAPI
E
DIC
F
DAPI
Vn
Gn
G
5mdC
H
5mdC/ DAPI
Vn
Gn
I
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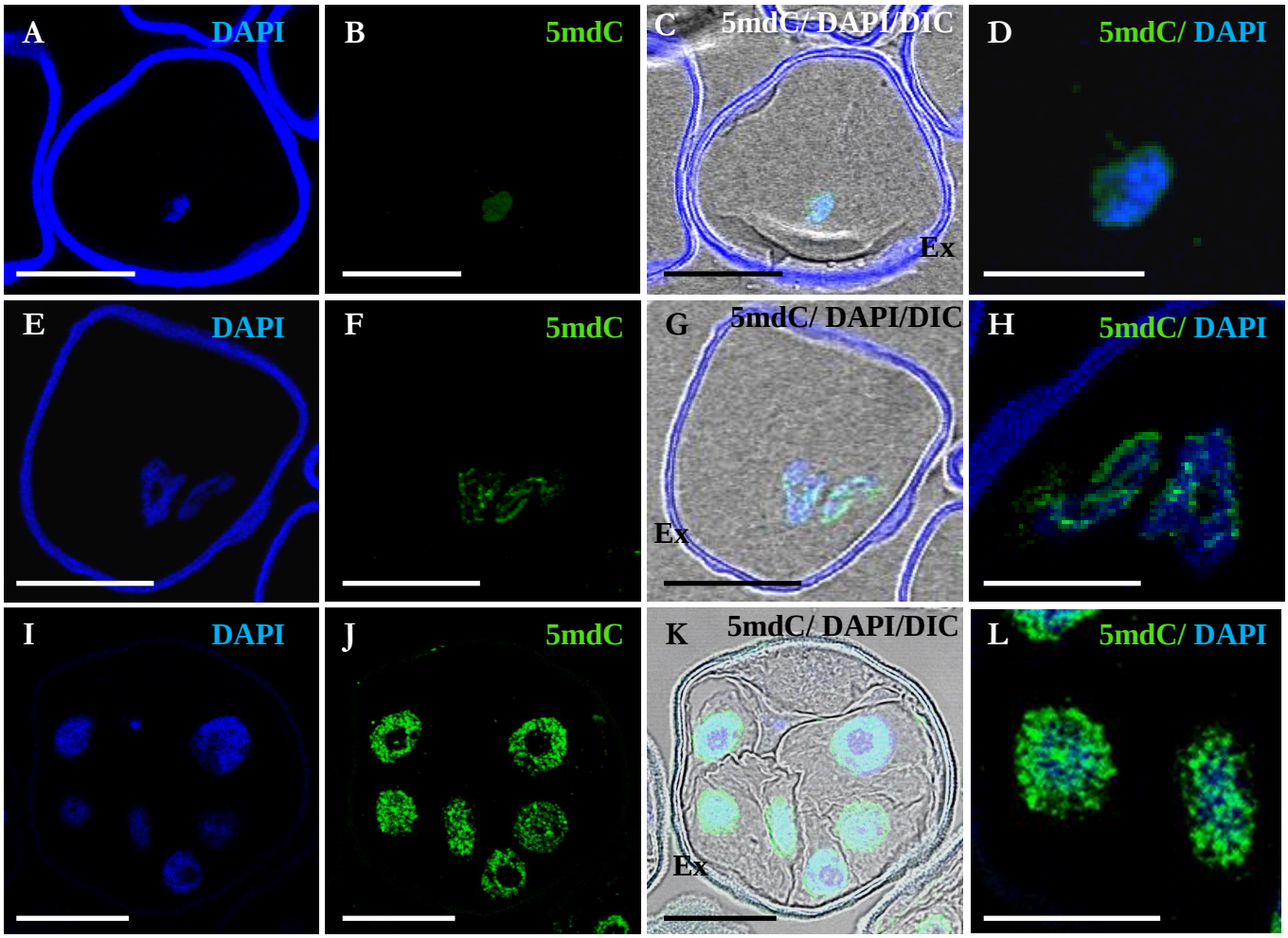
J
DAPI
Vn
K
5mdC
Vn
L
5mdC/ DAPI
Vn

Figure 4


A
DAPI
B
5mdC
C
5mdC/ DAPI/DIC
Ex
D
5mdC/ DAPI
E
DAPI
F
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G
5mdC/ DAPI/DIC
Ex
H
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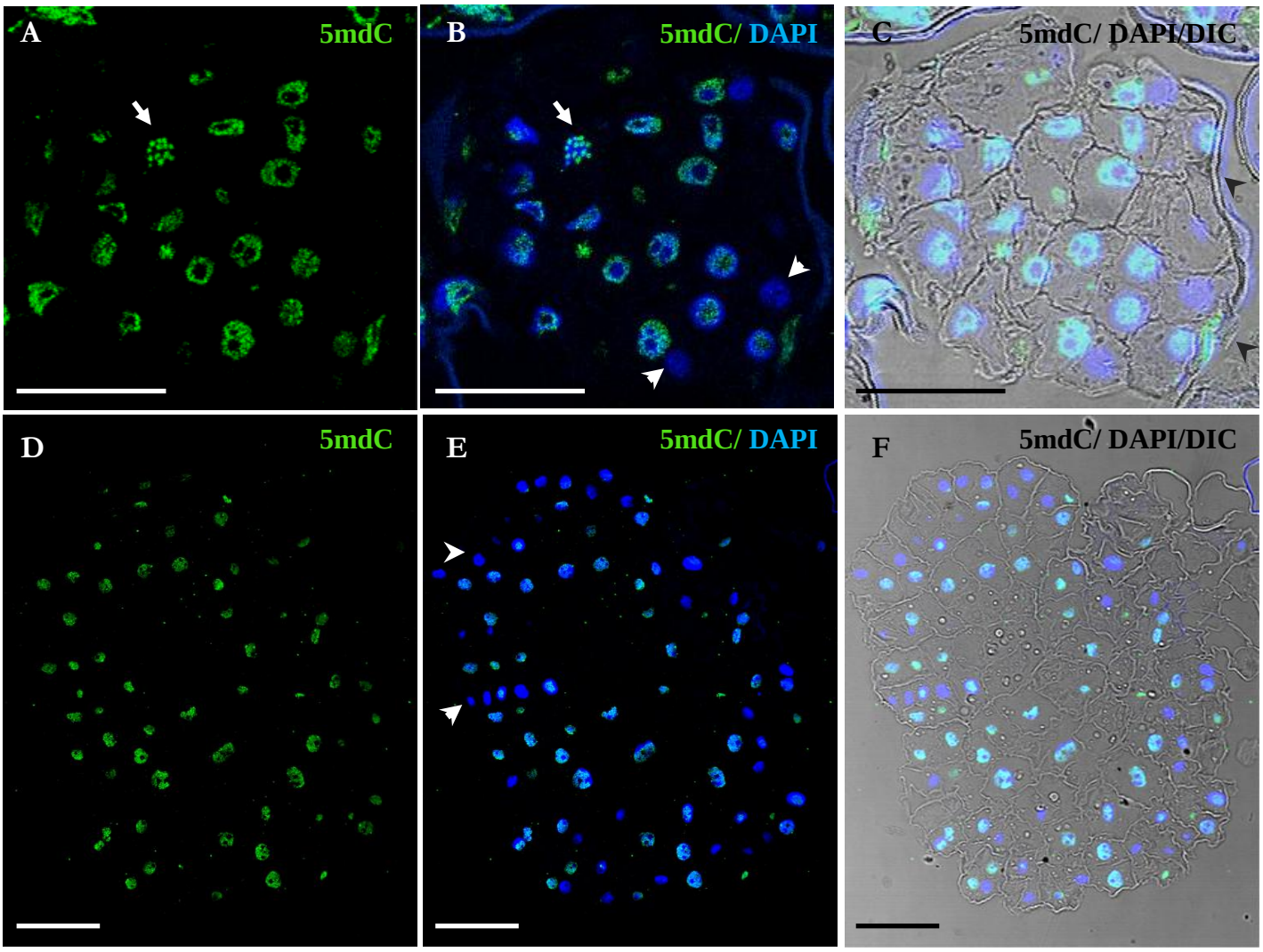
I
DAPI
J
5mdC
K
5mdC/ DAPI/DIC
Ex
L
5mdC/ DAPI

Figure 5


A
5mdC
B
5mdC/ DAPI
C
5mdC/ DAPI/DIC
D
5mdC
E
5mdC/ DAPI
F
5mdC/ DAPI/DIC