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DNA methylation dynamics and *MET1a-like* gene expression changes during stress-induced pollen reprogramming to embryogenesis

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Abstract

Stress-induced plant cell reprogramming involves changes in global genome organization, being the epigenetic modifications key factors in the regulation of genome flexibility. DNA methylation, accomplished by DNA methyltransferases, constitutes a prominent epigenetic modification of the chromatin fibre which is locked in a transcriptionally inactive conformation. Changes in DNA methylation accompany the reorganization of the nuclear architecture during plant cell differentiation and proliferation. After a stress treatment, *in vitro*-cultured microspores are reprogrammed and change their gametophytic developmental pathway towards embryogenesis, the process constituting a useful system of reprogramming in isolated cells for applied and basic research. Gene expression driven by developmental and stress cues often depends on DNA methylation; however, global DNA methylation and genome-wide expression patterns relationship is still poorly understood. In this work, the dynamics of DNA methylation patterns in relation to nuclear architecture and the expression of Bn*MET1a-like* DNA methyltransferase genes have been analysed during pollen development and pollen reprogramming to embryogenesis in *Brassica napus* L. by a multidisciplinary approach. Results showed an epigenetic reprogramming after microspore embryogenesis induction which involved a decrease of global DNA methylation and its nuclear redistribution with the change of developmental programme and the activation of cell proliferation, while DNA methylation increases with pollen and embryo differentiation in a cell-type-specific manner. Changes in the presence, abundance, and distribution of Bn*MET1a-like* transcripts highly correlated with variations in DNA methylation. Mature zygotic and pollen embryos presented analogous patterns of DNA methylation and *MET1a-like* expression, providing new evidence of the similarities between both developmental embryogenic programmes.

Key words: Cell fate, differentiation, DNA methyltransferase, epigenetics, male gametophyte development, 5-methylcytosine, microspore culture, pollen embryogenesis, proliferation.

Introduction

The microspore under stress conditions *in vitro* can switch its developmental programme towards embryogenesis to form haploid and double-haploid embryos and plants (Bárány *et al.*, 2005; Prem *et al.*, 2012). The comparison of the gametophytic and embryogenic pathways of the microspore reveal subcellular rearrangements and processes occurring in plant differentiating cells

switched to proliferation (Bárány *et al.*, 2005; Testillano *et al.*, 2005). In *Brassica napus* L., a species considered as a model for microspore embryogenesis induction, an efficient stress is the 32 °C treatment (Prem *et al.*, 2012). Nevertheless, this process presents a low efficiency in many species because its regulatory mechanisms are not well known.

Dynamic changes between chromatin states are relevant in the transcriptional regulation during pollen development and reprogramming to embryogenesis (Testillano *et al.*, 2005). Globally, heterochromatin increases during cell differentiation and organ maturation, while it decreases during cell de-differentiation and reorganization process (Exner and Hennig, 2008). There is increasing evidence that numerous processes of development and differentiation in both plants and animals are accompanied of chromatin remodelling (Kouzarides, 2007). Epigenetic control plays an essential role in the process of cellular differentiation allowing cells to be reprogrammed in order to generate new differentiation pathways (Costa and Shaw, 2007). DNA methylation and histone modifications have been revealed as hallmarks that define the functional status of chromatin domains and confer the flexibility of transcriptional regulation necessary for plant development and adaptive responses to the environment (Grant-Downton and Dickinson, 2005; Vaillant and Pazkowski, 2007).

DNA methylation constitutes a prominent epigenetic modification of the chromatin fibre, which becomes locked in a transcriptionally inactive conformation, thus leading to gene silencing. 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). DNA methylation of cytosine residues is accomplished by DNA methyltransferases, which can transfer methyl groups from S-adenosylmethionine to the DNA. DNA methyltransferase 1 (MET1) has been reported as the CG maintenance methyltransferase in plants, based on sequence similarity to DNMT1, the orthologous mammalian maintenance methyltransferase. However, there is increasing evidence suggesting that MET1 might also have *de novo* methyltransferase activity (Gehring and Henikoff, 2007). In *Arabidopsis thaliana*, *MET1* genes constitute a small multigene family (Finnegan and Kovac, 2000) whereas in *Brassica* two *MET1* genes have been characterized from *Brassica rapa*, Br*MET1a*, which is expressed in vegetative and reproductive organs, and Br*MET1b*, only expressed in pistils (Fujimoto *et al.*, 2006).

Stress-induced plant cell reprogramming involves changes in global genome organization, being the epigenetic modifications key factors of genome flexibility (Arnholdt-Schmitt, 2004). Gene expression driven by developmental and stress cues often depends on histone modifications and DNA methylation (Chinnusamy and Zhu, 2009). Previous studies have demonstrated large-scale reorganization of chromatin by local changes in DNA methylation and histone H4 acetylation in vegetative and floral buds of azalea, where DNA methylation and H4 deacetylation act simultaneously and coordinately, restructuring the chromatin and regulating gene expression during floral differentiation (Meijón *et al.*, 2009, 2010). 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). However, the knowledge of the DNA methylation regulation during the gametogenesis and pollen embryogenesis is very limited.

The present work has analysed the dynamics of DNA methylation patterns in relation to nuclear architecture and the expression of the DNA methyltransferase 1 gene *MET1a* during pollen development and pollen reprogramming to embryogenesis, in *Brassica napus* L. The results show an epigenetic reprogramming after microspore induction to embryogenesis, which involves a significant decrease of DNA methylation with the developmental programme change that initiates a proliferation process while it increases with pollen and embryo differentiation in a cell-type-specific manner. Changes in the presence, abundance, and distribution of *MET1a* transcripts are highly correlated with variations in DNA methylation, suggesting the possible involvement of a MET1-like protein in the methylation of pollen and embryo cells characterized during development.

Materials and methods

Plant material and microspore culture

Brassica napus L. cv. Topas donor plants were grown under controlled conditions at 15/10 °C in a 16/8 light/dark cycle. Isolated microspore culture and embryogenesis induction was performed at 32 °C, as described by Prem *et al.* (2012).

Cryoprocessing for microscopy analysis

Freshly isolated microspores and samples from different culture times were collected and fixed overnight at 4 °C with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS, dehydrated through a methanol series by progressive lowering of temperature and embedded in Lowicryl K4M resin at –30 °C under UV irradiation in an automated freeze substitution device (Leica, Vienna). Semithin sections were collected on slides, stained with toluidine blue and observed under bright field microscopy.

Immunofluorescence

Immunolocalization of 5-methyldeoxycytidine (5-mdC) was performed as previously described (Testillano *et al.*, 2005; Testillano and Risueño, 2009). Lowicryl semithin sections were mounted on 3-aminopropyltriethoxysilane-coated slides and treated with 5% bovine serum albumin (BSA) in PBS, for 10 min, denatured with 2 M HCl for 45 min and washed in PBS, incubated with anti-5-mdC mouse antibody (Eurogentec) diluted 1/50 in 1% BSA and Alexa-Fluor-488 anti-mouse antibody (Molecular Probes) diluted 1/25. Sections were counterstained with 1 mg ml^{-1} DAPI (4',6-diamidino-2-phenylindole) for 10 min and analysed by confocal microscopy (TCS-SP5, Leica).

As negative controls, DNA denaturation was avoided and immunodepletion was carried out by preblocking the antibody with 5-mdC at 4 °C overnight.

Quantification of global DNA methylation by high-performance capillary electrophoresis

Freshly isolated microspores and embryo samples from different culture times were collected and frozen in liquid nitrogen. Genomic DNA was extracted from 100 mg freshweight using a plant genomic DNA extraction kit (DNeasy Plant Mini, Qiagen) according to the manufacturer's instructions and adding 10 µl RNase A (Qiagen). DNA was concentrated into DyNA Vap (Labnet) and resuspended in ddH$_2$O to a concentration of 1 µg µl^{-1}. DNA samples (5 µl) were denatured by heating for 2 min

and cooling rapidly in ice. Then, 0.25 µl of 10 mM $ZnSO_4$ and 0.5 µl of nuclease P1 (200 U/ml in 30 mM $C_2H_3O_2Na$, Sigma) were added. Mixtures were incubated for 16 h at 37 °C. Next, 0.5 µl TRIS (0.5 M, pH 8.3) and 0.25 µl alkaline phosphatase (50 U ml^{-1} in 2.5 M $(NH_4)_2SO_4$, Sigma) were added and the mixtures were incubated for 2 h at 37 °C. Hydrolysed solutions were centrifuged for 20 min at 15,000 g. Samples were analysed according to Fraga *et al.* (2002) with the modifications of Hasbún *et al.* (2008) by high-performance capillary electrophoresis (HPCE) using capillary ion analysis (Waters Chromatography) and a PACE 2050 (Beckman) capillary electrophoresis instrument in which the conditions of separation and capillaries were adjusted according to Cifuentes *et al.* (1999) and the instrument.

Three biological and two analytical replicates per sample were taken. Quantification of the relative methylation of each sample was performed as the percentage of 5-mdC peak of total deoxycytidines (dC + 5-mdC) peaks. *P*-values were calculated using the Student t-test.

Reverse-transcription PCR

RNA was isolated with RNeasy Plant Micro and RNeasy Plant Mini kits (Qiagen) for the first culture stages, and with the acid guanidine thiocyanatephenol/chloroform method using the Trizol reagent kit for the advanced culture stages. Total RNA (1 µg) was used for reverse transcription using the Superscript TM II reverse transcriptase (Invitrogen). The oligonucleotides used for Bn*MET1a* expression analysis were: 5'-GGCAGACGTTCCAACTTACT-3'-and 3'-AAGGTGCACCGTAT TGAGTA-5' from the sequence of Br*MET1a* (accession AB251937), one of the MET1 DNA methyltransferases characterized in *B. rapa* (Fujimoto *et al.*, 2006). cDNA was amplified by PCR using the Hot Master Taq polymerase (Eppendorf). Amplification products were detected on 1% agarose gels stained with ethidium bromide. Band intensity was expressed as relative absorbance units. Each cDNA band density was first normalized by dividing it by the density of the actin II band in the same lane.

Fluorescence in situ *hybridization*

Fixed samples were cryoprotected in 2.3 M sucrose. Small samples from the first culture stages were cryofixed in liquid propane and cryosectioned (Tokuyasu, 1973), whereas large samples of the last stages were embedded in OTC, frozen on dry ice, and sectioned in a cryostat.

cDNA was obtained as described before and used for PCR amplification, with the Br*MET1a* primers mentioned above. The amplified fragments were isolated from agarose gels and cloned using a pGEMT-Easy cloning system (Promega). DIG-RNA-MET1 probes were generated by *in vitro* transcription using the DIG-RNA labelling kit (Roche).

Thick cryostat sections were permeabilized by dehydration–rehydration in a methanol series and treatment with 2% cellulase (Onozuka R-10) for 1 h, washed, and dried. Semithin cryosections were washed in PBS to remove the sucrose and dried.

RNA/RNA fluorescence *in situ* hybridization (FISH) was performed as described (Testillano and Risueño, 2009) using dig-MET1a RNA probes diluted 1/50 in hybridization buffer at 50 °C overnight. Post-hybridization washes were performed in 4 × SSC, 2 × SSC, and 0.1 × SSC. Hybridization signal was detected by incubation with mouse anti-digoxigenin antibodies (1:5000 in 1% BSA, Sigma) for 90 min, followed by Alexa-Fluor-488 anti-mouse antibody (1:25 in PBS for 45 min, Molecular Probes). After washing in PBS, sections were counterstained with DAPI, mounted in Mowiol, and observed by confocal microscopy. Controls were performed with the sense probe.

Results

Global DNA methylation levels and 5-mdC distribution pattern during pollen development and after pollen reprogramming to embryogenesis

During pollen development, microspores underwent a vacuolation process, presenting a large vacuole which pushed the nucleus to a peripheral location (Fig. 1A). After the first pollen mitosis, the small generative cell exhibited a highly condensed chromatin, while the vegetative cell showed less condensed chromatin and a cytoplasm with storage products and small vacuoles (Fig. 1B). The application of a stress treatment to the vacuolated microspore changes the gametophytic pathway to an embryogenic
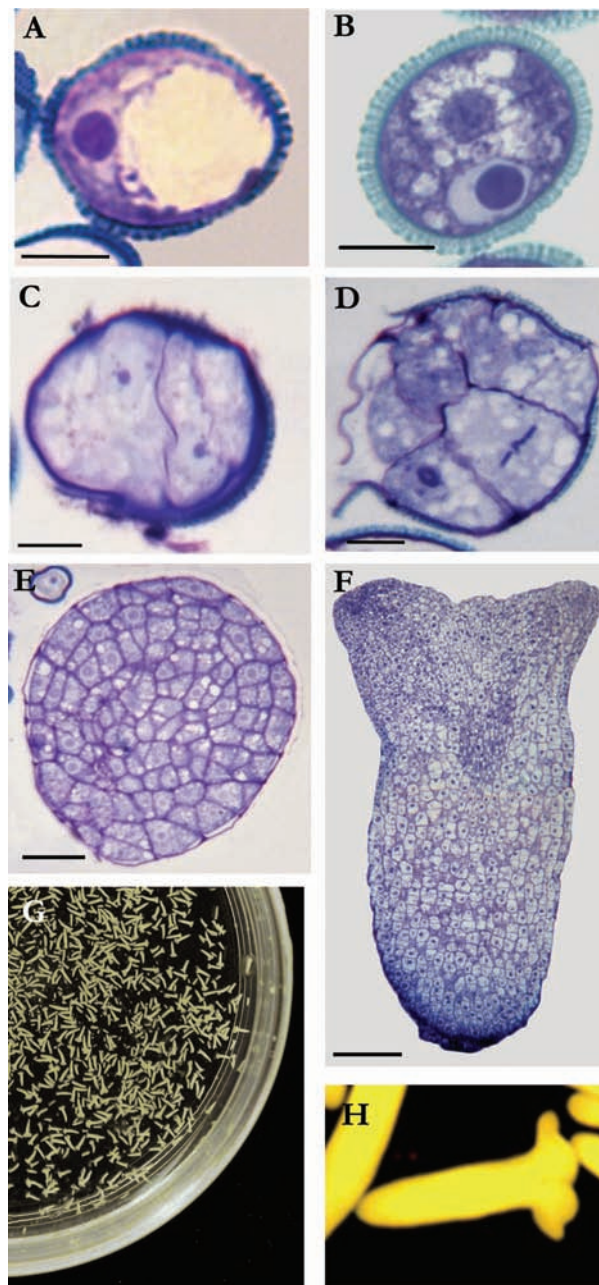

Fig. 1. Gametophytic and embryogenic pollen development. (A–F) Semithin sections stained with toluidine blue showing pollen gametophytic development *in vivo* (A, B) and pollen embryogenic development *in vitro* (C–H). (A) Vacuolated microspore. (B) Mature pollen. (C) Two-cell pro-embryo. (D) Multicellular pro-embryo. (E) Globular embryo. (F) Torpedo embryo. (G, H) Cotyledonary embryos: panoramic and detailed views. Bars, 10 µm (A–D), 20 µm (E), 50 µm (F) (this figure is available in colour at JXB online).

one, with the formation of haploid embryos. The embryogenic microspores underwent a symmetrical cell division giving rise to 2-cell pro-embryo structures (Fig. 1C) with nuclei similar in size and organization, in contrast with the bicellular pollen (Fig. 1B) following gametophytic development. Subsequent divisions led to multicellular pro-embryos, confined inside the exine (Fig. 1D). At later stages, when the exine broke down, cell proliferation increased, and later globular embryos were observed (Fig. 1E), formed by small polygonal cells with large central nuclei. As embryogenesis progresses, embryos elongated giving rise to heart-shaped and torpedo embryos (Fig. 1F), that finally increased their size and lead to the formation of mature cotyledonary embryos (Fig. 1G, 1H).

Quantification of global DNA methylation and immunofluorescence of 5-mdC were performed at different stages of pollen development (vacuolated microspore and mature pollen) and after pollen reprogramming to embryogenesis, in early multicellular pro-embryos. Capillary electrophoresis presents a powerful analytical method to provide very useful information on an organism's composition, such as nucleic acids, proteins, and metabolites (Castro-Puyana *et al.*, 2012). HPCE results showed a low level of DNA methylation in vacuolated microspores (3.02%) and a significant increase during pollen maturation, with 11.66% of methylated cytosine in mature pollen (Fig. 2A). However, after pollen reprogramming to embryogenesis, DNA methylation in early pro-embryos was 4.33%, a value which did not differ significantly from the vacuolated microspore (3.02%) (Fig. 2A).

Confocal microscopy analysis of 5-mdC immunofluorescence assays revealed the nuclear distribution of methylated DNA and showed specific changes in the distribution pattern of 5-mdC during the developmental processes analysed. In the vacuolated microspore, 5-mdC immunofluorescence showed a punctuate pattern with very few small spots at the periphery of the nucleus (Fig. 2C, C'), while in mature pollen, 5-mdC signal was intense over the whole area of the generative nucleus, associated with its highly condensed chromatin, and much lower in the vegetative nucleus (Fig. 2B, B'). On the contrary, after reprogramming, early pro-embryo cells showed a similar pattern of 5-mdC distribution than vacuolated microspores (Fig. 2D, D'). Negative controls performed either by immunodepletion (Fig. 2E, 2G) or by avoiding DNA denaturation of the section (Fig. 2F) did not show any fluorescent signal. Exine showed unspecific autofluorescence in most structures (Fig. 2B', C', D', E, F, G).

Global DNA methylation levels and 5-mdC distribution pattern during pollen embryogenesis progression and in zygotic embryos

Four representative consecutive stages of the pollen embryo development were analysed: the initial stage of vacuolated microspore, early multicellular pro-embryos, globular embryos, and cotyledonary embryos (Figs. 1A, 1C, 1E, 1H). HPCE analysis showed a progressive increase on DNA methylation levels (Fig. 3A) during the process. As mentioned above, early pro-embryos presented low DNA methylation levels (4.33%), similar to vacuolated microspores (3.02%), while globular embryos

displayed a significant increase on methylation levels (6.67%). The highest DNA methylation levels were registered in cotyledonary embryos (12.27%).

Confocal analysis of 5-mdC immunolocalization showed different patterns of distribution in different cell types of developing pollen embryos. At early stages, nuclei of multicellular pro-embryos showed a punctuate pattern of 5-mdC at the periphery of the nucleus (Fig. 2D, D'). At later stages, in globular (Fig. 3B, 3C) and torpedo (data not shown) embryos, the differentiated cells, located in the outer layers that forms the epidermis and underlying cells, showed larger and more abundant fluorescent spots (Fig. 3B), indicating a higher presence of 5-mdC in nuclei of differentiating cells than in proliferating cells, located in the inner part of the embryo (Fig. 3C). In torpedo and cotyledonary embryos, this differential 5-mdC pattern was also present (Fig. 3D, 3F). The most intense immunofluorescent signal was found in the differentiated cells (Fig. 3D) while in the proliferating cells, the 5-mdC signal was lower (Fig. 3F).

Mature zygotic embryos obtained from seeds were also analysed for comparison. Results showed that zygotic embryos had a similar 5-mdC pattern than pollen embryos, with a differential distribution between differentiated (Fig. 3E) and proliferating (Fig. 3G) cells, the higher fluorescent 5-mdC signal was found in the differentiated cells of the peripheral cell layers and cotyledonary regions (Fig. 3E) whereas a lower 5-mdC signal was observed in meristematic regions (Fig. 3G).

*Bn*MET1a *expression during pollen gametophytic development and after pollen reprogramming to embryogenesis*

The expression of DNA methyltransferase-like genes of MET1 type was analysed by fluorescence *in situ* hybridization (FISH) and reverse-transcription PCR (RT-PCR) analysis, at different stages of pollen development and after pollen reprogramming. RT-PCR analysis of the Bn*MET1a-like* sequence showed that expression was developmentally upregulated during pollen maturation, while it kept low levels after pollen reprogramming (Fig. 4A). In mature pollen, Bn*MET1a* expression was 2-fold higher than in vacuolated microspores, whereas after reprogramming, early pro-embryos showed expression values similar to vacuolated microspores (Fig. 4A).

To analyse the spatial expression pattern and the subcellular distribution of Bn*MET1a* transcripts, FISH was carried out, followed by confocal microscopy analysis. In vacuolated microspores, there was a low hybridization signal in the peripheral layer of cytoplasm while nuclei, vacuoles, and walls appeared negative (Fig. 4C, C'). In mature pollen, high fluorescent signal was observed in the cytoplasm (Fig. 4B, B') corresponding to abundant transcripts in the cytoplasm, while vegetative (large) and generative (small) nuclei of mature pollen did not show any FISH signal. In contrast, after reprogramming the fluorescence hybridization signal in the cytoplasm of early pro-embryos was considerably reduced (Fig. 4D, D'). No hybridization signal was observed in control experiments with the sense probe in all stages (Fig. 4E, 4F, 4G).
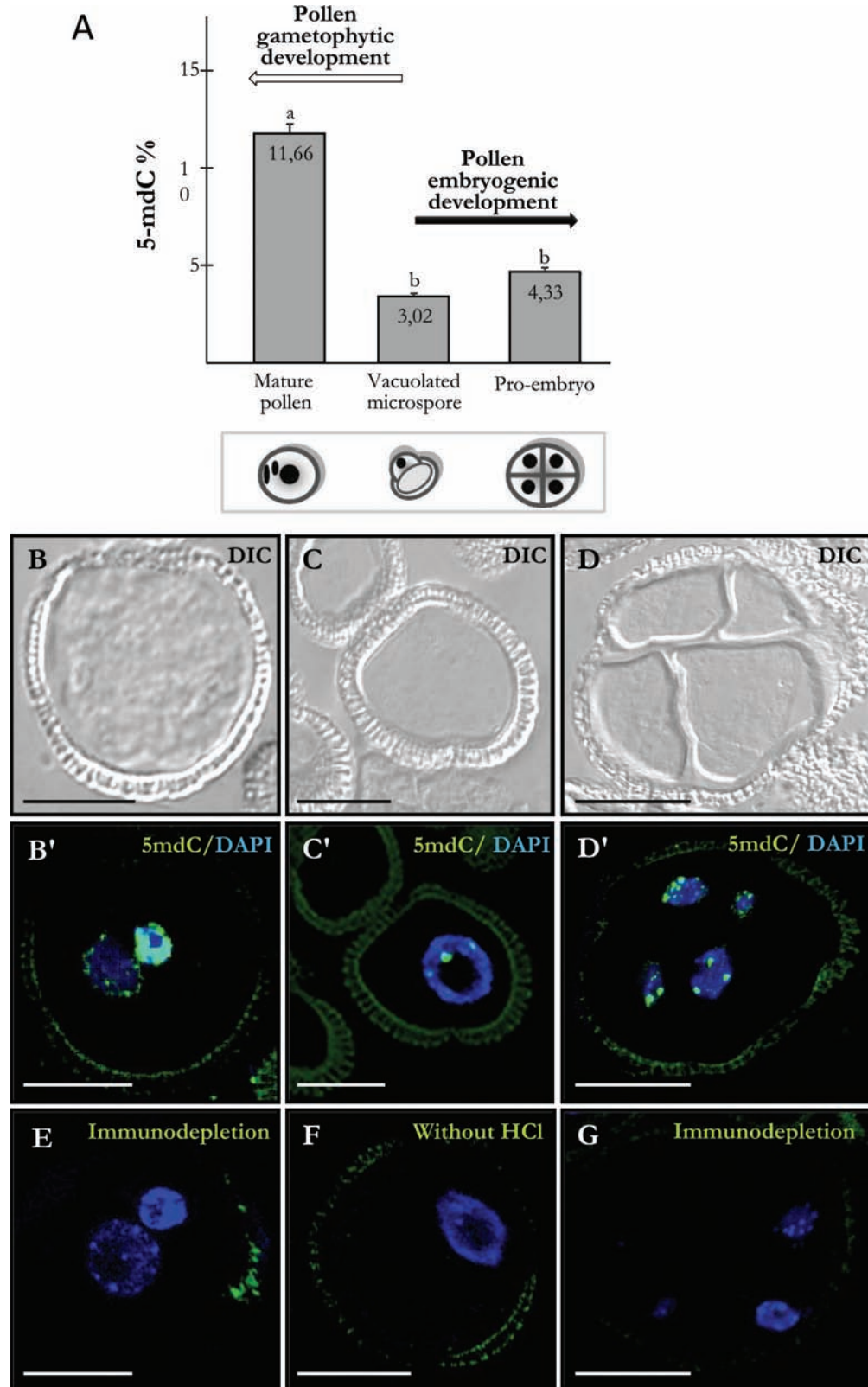
Fig. 2. Quantification of DNA methylation by high-performance capillary electrophoresis and 5-methyldeoxycytidine (5-mdC) immunolocalization during pollen development and after pollen reprogramming. (A) Percentage of 5-mdC at different developmental stages. Lower-case letters indicate significant differences at $P < 0.001$. (B–G) Differential interference contrast (DIC) images or merged images of 5-mdC immunofluorescence (green) and DAPI staining of nuclei (blue). B, C, and D are the same structures as B', C', and D', respectively. (B, B') Mature pollen. (C, C') Vacuolated microspore. (D, D') Four-cell pro-embryo confined by the exine. (E, G) Negative controls by immunodepletion in mature pollen (E) and 4-cell proembryo (G). (F) Negative control avoiding DNA denaturation by HCl in a vacuolated microspore. Bars, 10 μm (B, B', C, C', E, F), 20 μm (D, D', G).
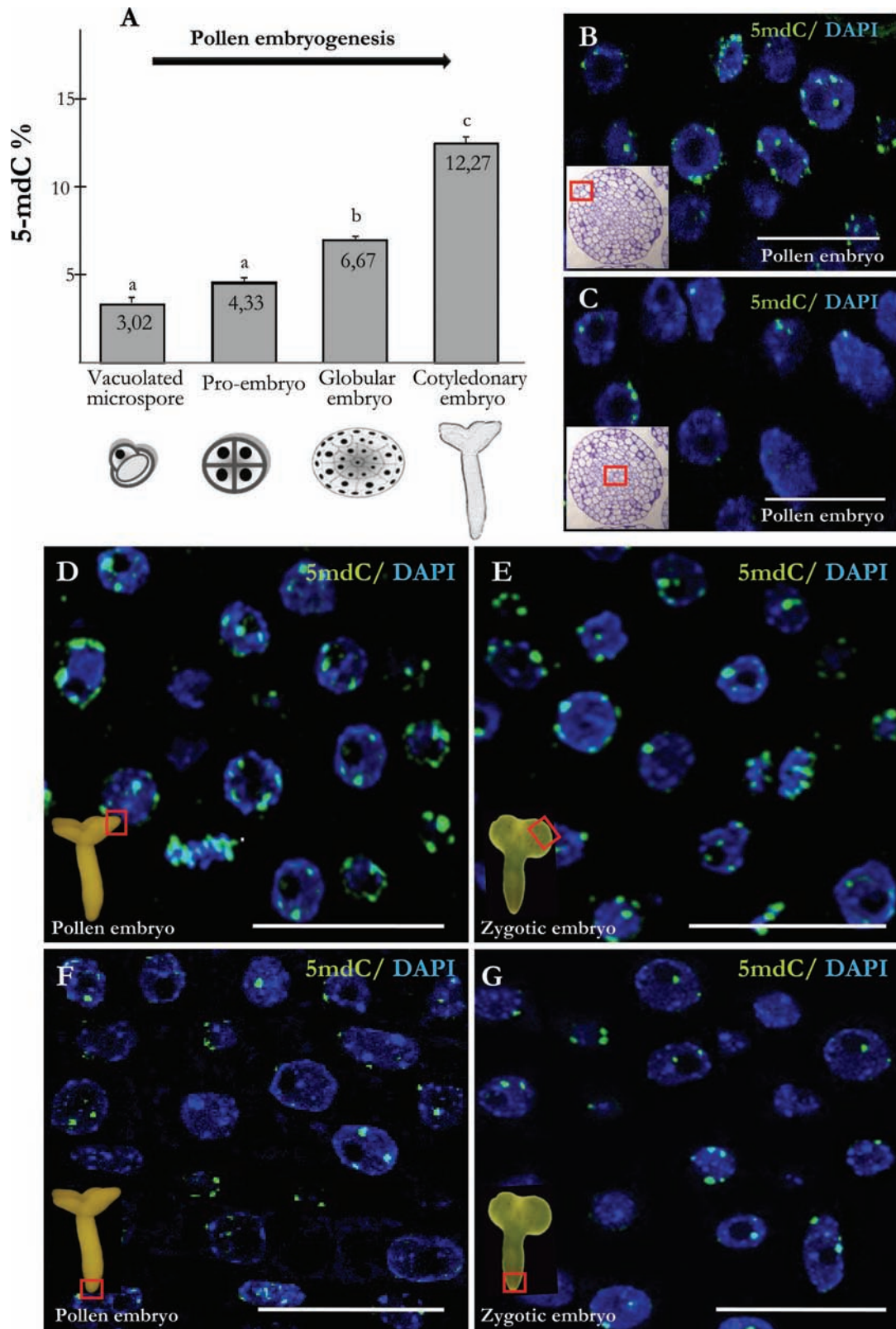
Fig. 3. Quantification of DNA methylation by high-performance capillary electrophoresis and 5-methyldeoxycytidine (5-mdC) immunolocalization in pollen embryogenesis and zygotic embryos. (A) Percentage of 5-mdC at different stages of pollen embryogenesis. Lower-case letters indicate significant differences at *P* < 0.001. (B–G) Merged images of 5-mdC immunofluorescence (green) and DAPI staining of nuclei (blue) in differentiating (B, D, E) and proliferating (C, F, G) cells of globular (B, C) and cotyledonary (D, F) pollen embryos and cotyledonary zygotic embryos (E, G). Fields of view are indicated by the squares in the insets. Insets in B, C: toluidine blue-stained sections of pollen globular embryos. Bars, 20 µm (B, C), 25 µm (D, E, F, G).
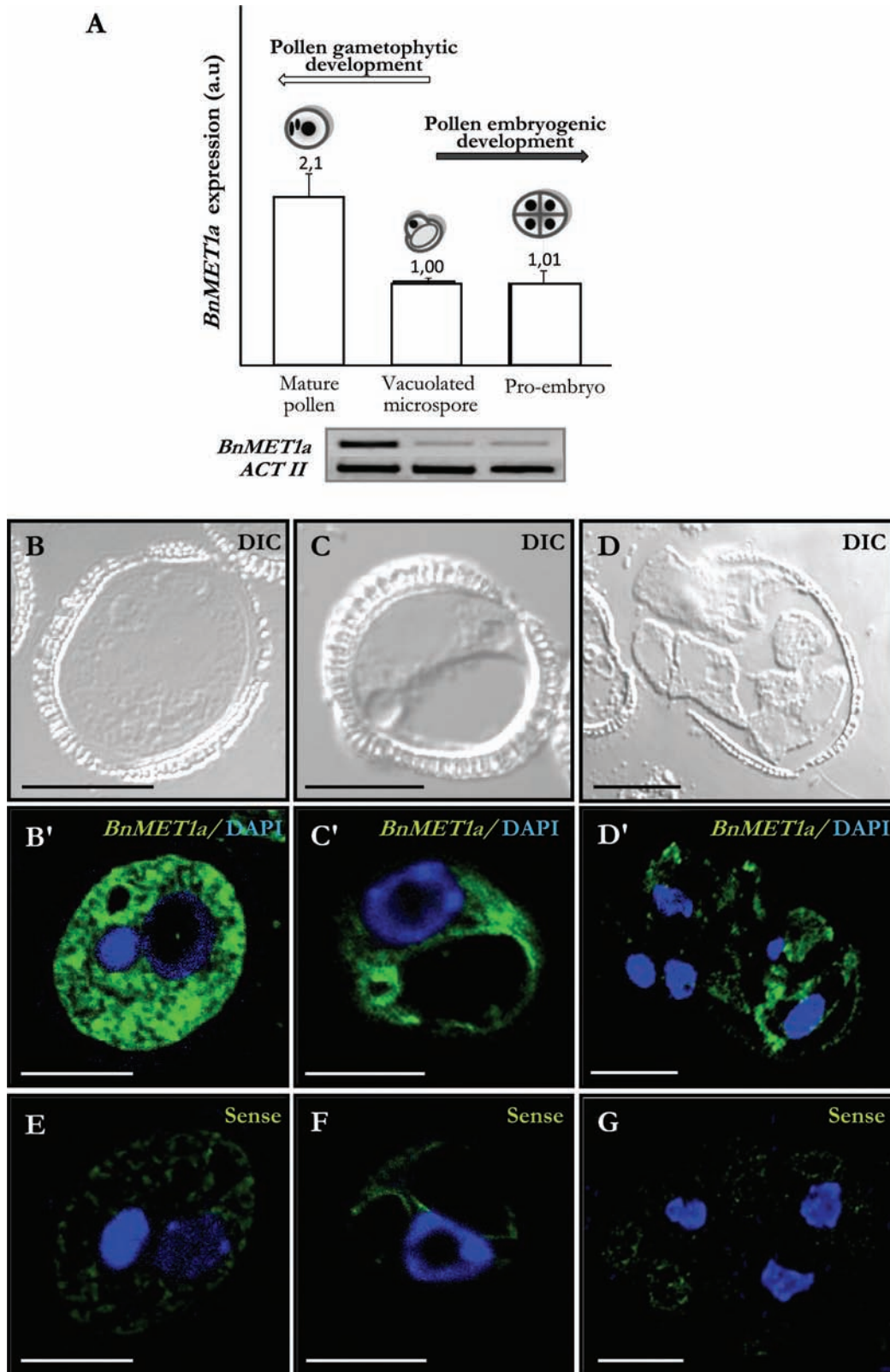
Fig. 4. Bn*MET1a-like* expression during pollen development and after pollen reprogramming. (A) Reverse-transcription PCR relative band intensities in arbitrary units (a.u.) and representative electrophoresis gels of the amplification products. (B–G) FISH localization of Bn*MET1a* transcripts. Differential interference contrast (DIC) images and merged images of FISH signals (green) and DAPI staining of nuclei (blue). B, C, and D are differential interference contrast images of the same structures as B', C', and D', respectively. (B, B') Mature pollen. (C, C') Vacuolated microspore. (D, D') Multicellular pro-embryo with exine. (E–G) FISH controls with the sense probe in mature pollen (E), vacuolated microspore (F), and multicellular pro-embryo (G). Bars, 10 μm (B, B', C, C', E, F), 20 μm (D, D', G).

BnMET1a expression during pollen embryogenesis progression and in zygotic embryos

RT-PCR analysis showed that during the progression of pollen embryogenesis, the Bn*MET1a* expression pattern changed. Bn*MET1a* expression was low at early stages (vacuolated microspore and multicellular pro-embryos), while at later stages expression was induced: the expression levels increased with differentiation in globular and cotyledonary embryos (Fig. 5A).

Confocal analysis of FISH preparations showed a low hybridization signal in the cytoplasm of early pro-embryo cells (Fig. 5B, B'), whereas in globular embryos the fluorescent signal was higher, especially in the differentiating outer cells (Fig. 5C, C'). The signal was specifically found in the cytoplasm, where transcripts were localized, while no signal was found in nuclei, vacuoles, or cell walls (Fig. 5D). In globular, torpedo, and cotyledonary microspore embryos, a differential Bn*MET1a* expression pattern was found. Differentiating cells of peripheral regions presented a higher hybridization signal than proliferating cells of embryo meristems (Fig. 6A, 6C). Control FISH experiments performed with sense probes did not show fluorescence in any sample.

Expression analysis was also carried out in cotyledonary zygotic embryos. FISH results showed that Bn*MET1a* has a similar expression pattern in both types of embryo (Fig. 6A–D), and RT-PCR analysis did not show significant differences in Bn*MET1a* expression between cotyledonary pollen and zygotic embryos (Fig. 6E).

Discussion

Pollen reprogramming is associated with the decrease of global DNA methylation and its nuclear redistribution

This study analyses DNA methylation pattern dynamics in relation to the nuclear architecture during pollen development and reprogramming towards embryogenesis and shows that both processes are epigenetically regulated. Differences in global DNA methylation levels and changes in the 5-mdC distribution pattern were revealed in the two pollen pathways (gametophytic development and pollen embryogenesis). High levels of global DNA methylation were characteristic of pollen differentiation, whereas low levels of global methylation were found after pollen reprogramming, in proliferating cells.

Several studies have described an increase on global DNA methylation levels with the differentiation of plant reproductive organs (Zluvova *et al.*, 2001; Meijón *et al.*, 2009), during the formation of the oak pollen (Ribeiro *et al.*, 2009), and during the dormant period of the vegetative buds in *Castanea sativa* (Santamaría *et al.*, 2009). However, the nuclear distribution pattern of the methylated DNA has not been analysed in those systems. The current study revealed changes in the 5-mdC distribution pattern that are related to variations in global DNA methylation, which subsequently are reflected in changes in the transcriptional activity.

The punctuate 5-mdC distribution nuclear pattern and low DNA methylation levels of the vacuolated microspore are in agreement with a high transcriptional activity and a decondensed chromatin pattern, as reported for the vacuolated microspore nuclei (Seguí-Simarro *et al.*, 2011). However, as the pollen development progressed, the vegetative and the generative nuclei of mature pollen showed a very different 5-mdC distribution pattern corresponding to the distinct chromatin condensation level of each nucleus. Large-scale chromatin modifications have been recently associated with the differentiation of gametophytic cells (Baroux *et al.*, 2011). The highly condensed chromatin pattern of the generative nucleus is related to a very low or absent transcriptional activity, while the organization of the vegetative nucleus is related to a higher transcriptional activity state (McCormick, 1993; Testillano *et al.*, 2005). Ultrastructural and cytochemical analysis of *Brassica napus* pollen grains has revealed large masses of highly condensed chromatin and scarce interchromatin structures in the generative nucleus, whereas the vegetative one showed chromatin decondensation and small chromatin masses (Seguí-Simarro *et al.*, 2011). According to the current results, the increase on the global DNA methylation levels during the pollen development is associated with the heterochromatization process of the generative and sperm nuclei that happens with the differentiation process. The vegetative nucleus exhibited much less methylated DNA, which fits well with a higher chromatin transcription activity to synthesize products necessary for pollen tube germination.

The current study shows that pollen reprogramming to embryogenesis, in contrast with the gametophytic development, is associated with the decrease of global DNA methylation. Cell reprogramming by stress involves morphological and physiological changes as well as modifications in the genome organization, so that it requires specific factors that contribute in the regulation of genome flexibility, epigenetic modification being a key factor in this flexibility (Arnholdt-Schmitt, 2004; Miguel and Marum, 2011). The current study indicates a distribution pattern of 5-mdC on small condensed chromatin masses at the nuclear periphery in early pro-embryos cells. The chromatin condensation level of the pro-embryo nuclei has been reported as similar to that in cycling cells in several species (González-Melendi *et al.*, 1998), including *Brassica napus* (Seguí-Simarro *et al.*, 2011), indicating that pollen pro-embryo cells are proliferating at the beginning of the embryogenesis.

The data reveal an epigenetic change associated with the change of developmental programme and with the activation of cell proliferation occurring at the beginning of embryogenesis, which would be related to a global change of gene expression (Reik *et al.*, 2001; Meijón *et al.*, 2010) as revealed by transcriptomic analysis (Maraschin *et al.*, 2006). This epigenetic change is associated with the acquisition of embryogenic competence by the microspore. Reports in tree species have showed that the morphogenic capacity is associated with low levels of DNA methylation (Valledor *et al.*, 2010) and a transient DNA demethylation of the ovules occurred after pollination (Viejo *et al.*, 2010).

The epigenetic changes observed after pollen reprogramming to embryogenesis could lead to an increase of the cellular plasticity by facilitating the access of transcription factors to the chromatin fibre. According to Costa and Shaw (2006, 2007), the ability of remodelling the chromatin organization may provide
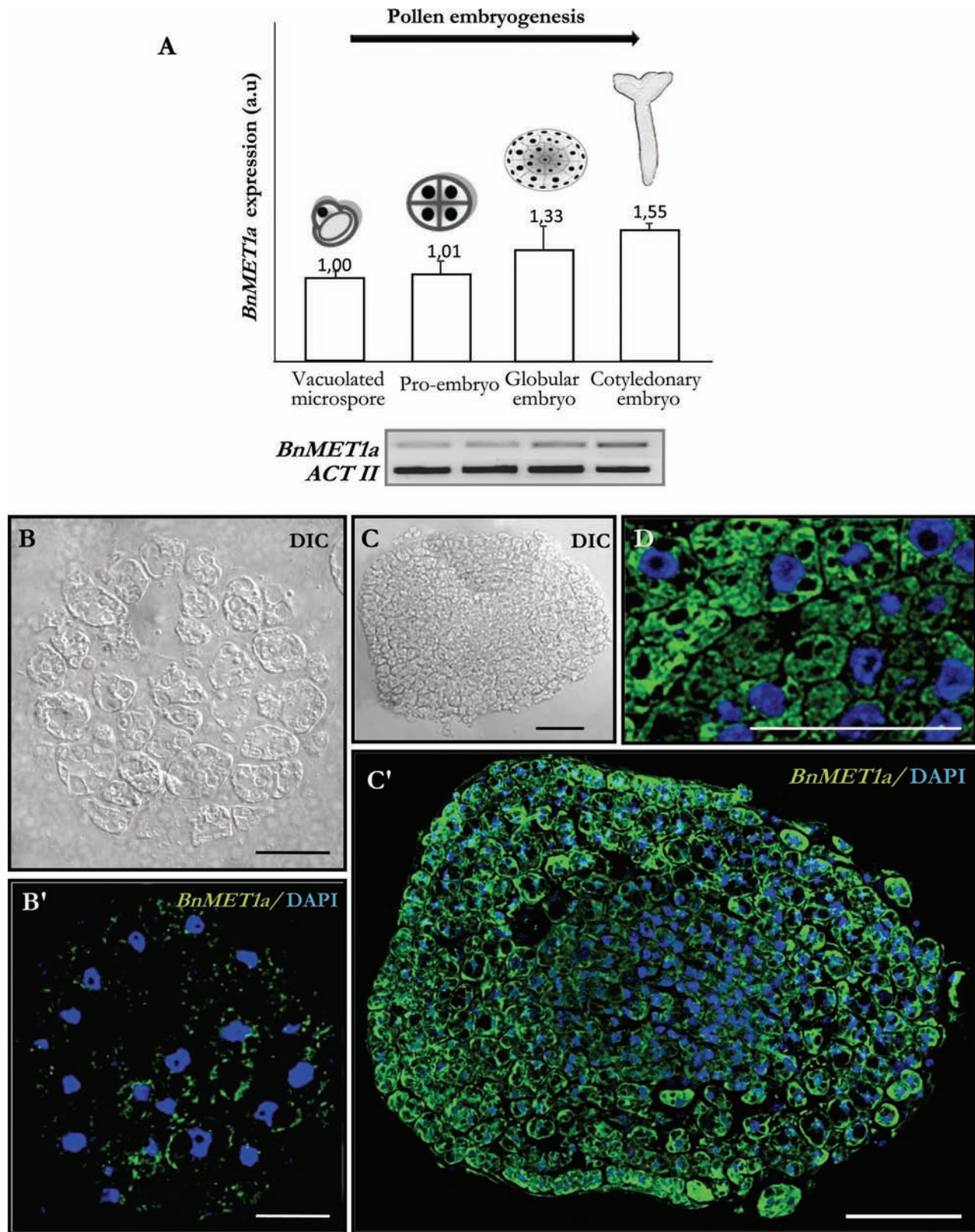
Fig. 5. Bn*MET1a-like* expression during pollen embryogenesis. (A) Reverse-transcription PCR relative band intensities in arbitrary units (a.u.) and representative electrophoresis gels of the amplification products. (B, B', C, C', D) FISH localization of Bn*MET1a* transcripts. Differential interference contrast images (B, C) and merged images (B', C', D) of FISH signals (green) and DAPI staining of nuclei (blue). B and C are the same structures as B' and C'. (B, B') Multicellular pro-embryo. (C, C') Late globular embryo. (D) Higher magnification of a globular embryo region. Bars, 20 μm (B, B'), 75 μm (C, C', D).
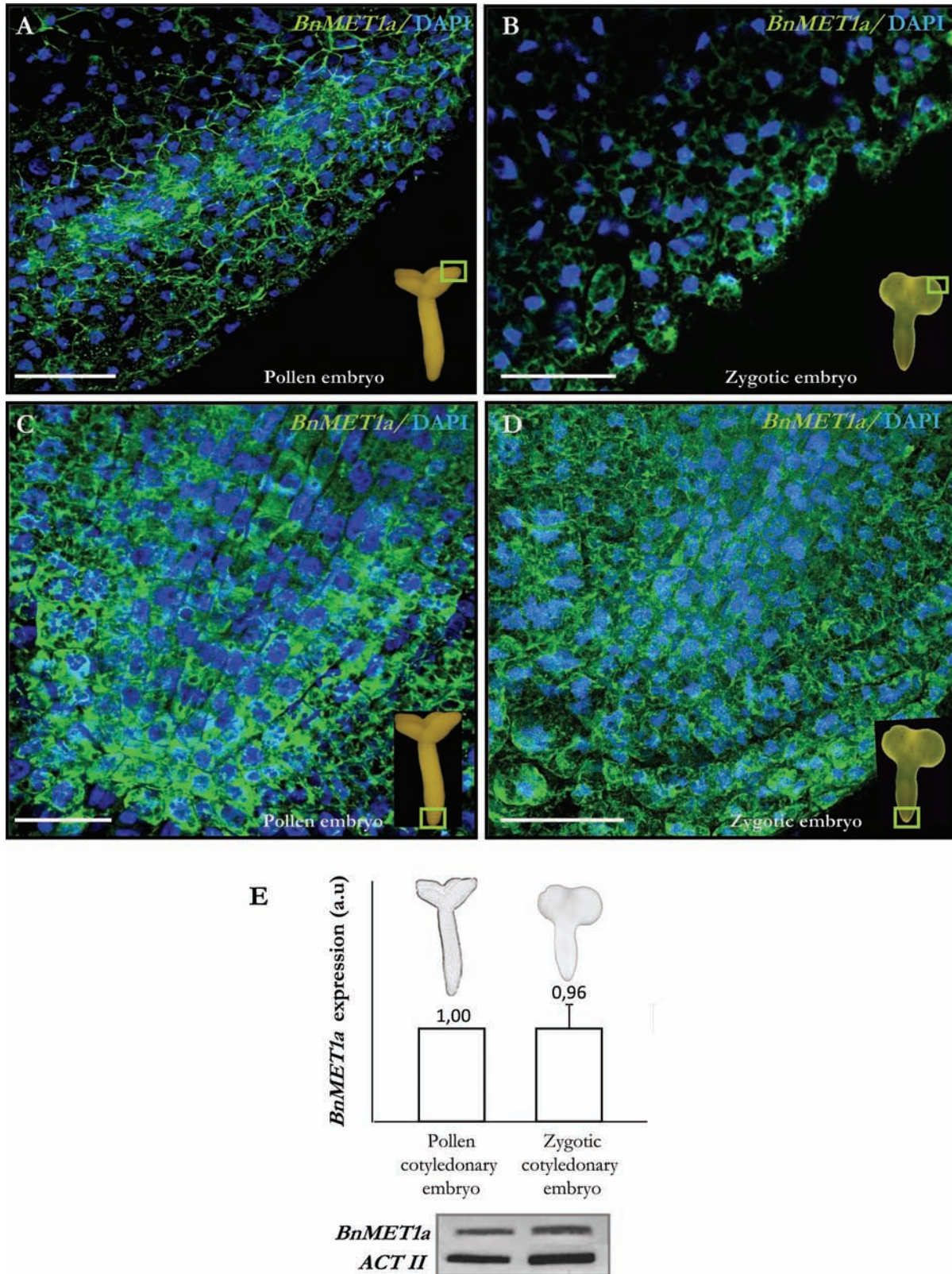
Fig. 6. Bn*MET1a-like* expression in cotyledonary pollen and zygotic embryos. (A–D) Merged images of FISH signals (green) and DAPI staining of nuclei (blue) of cotyledon (A, B) and radical (C, D) regions in pollen embryos (A, C) and zygotic embryos (B, D). Fields of view are indicated by the squares in the insets. Bars, 50 µm. (E) Histogram showing reverse-transcription PCR relative band intensities in arbitrary units (a.u.) and representative electrophoresis gels of the amplification products.

the base of the plasticity of changes in cellular fate and cellular totipotency as an additional platform at the level of the regulation of genetic information. The current results demonstrate that epigenetic control through the DNA methylation plays an important role in the reprogramming process and cellular differentiation, suggesting that the decrease of global methylation levels would allow the cells to be reprogrammed. Thus, the results indicate, as far as is known for the first time, the existence of an epigenetic reprogramming after pollen embryogenesis induction.

Pollen embryogenesis progression involves an increase in global DNA methylation associated with cell differentiation

During advanced stages of pollen embryogenesis, this study observed an increase in global DNA methylation levels that was associated with differentiation events at specific developmental stages. Differentiation started with the protodermis formation in globular embryos which showed a moderate increase of DNA methylation, whereas the highly differentiated cotyledonary embryos exhibited the highest methylation levels. These results indicate that the progress of the cellular differentiation is related to a fast increase of global DNA methylation levels in pollen embryos, as found in other systems (Costa and Shaw, 2007).

The results show a punctuate nuclear distribution pattern of 5-mdC associated with peripheral condensed chromatin masses which varied in size and number in a cell type specific manner. The 5-mdC signal increased with embryogenesis progression with a differential distribution among cells, proliferating cells presenting weaker 5-mdC signals, while differentiating cells presented more intense signals. Chromatin organization changes have been reported at early stages of pollen embryogenesis in several species (Testillano *et al.*, 2000, 2005) and *Brassica napus* (Seguí-Simarro *et al.*, 2011). Nevertheless, until now no data have been available on the advanced stages of pollen embryo development. The increase of global methylation observed during embryogenesis was associated with the heterochromatization that accompanied cellular differentiation in the most advanced stages. These data indicate an epigenetic control during pollen embryo development.

*Bn*MET1a-like *expression changes at transcriptional level during pollen reprogramming and embryogenesis*

The expression of DNA methyl-transferase-like genes of MET1 type in *Brassica napus* was approached by using the sequence of Br*MET1a* characterized in the highly homologous species *B. rapa* and expressed in vegetative and reproductive organs in *B. rapa* (Fujimoto *et al.*, 2006). The results provide evidence that Bn*MET1a-like* is expressed in *B. napus* pollen and embryos, changing its expression at transcriptional level during the gametophytic development and pollen embryogenesis. Bn*MET1a* was induced during pollen differentiation, while after pollen reprogramming, Bn*MET1a* expression was similar to the vacuolated microspore and increased at late embryogenesis stages. These modifications on Bn*MET1a* expression correlated highly with the changes in global DNA methylation during the two pollen developmental programmes, as summarized in Fig. 7.

MET1 homologues have been characterized in various plants, being expressed in vegetative and reproductive organs. In *B. rapa* two genes, Br*MET1a* and Br*MET1b*, have been identified, probably with different functions (Fujimoto *et al.*, 2006). The structural conservation of their amino acid sequences with At*MET1*, whose methyltransferase activity was proved in *Arabidopsis*, indicated that they may function as DNA methyltransferases (Fujimoto *et al.*, 2006). MET1a has been suggested as the most predominant methyltransferase regulating gene expression in various tissues in *B. rapa*, showing high *MET1a* expression in flower buds and increasing mRNA levels through flower development (Fujimoto *et al.*, 2006). Recent studies have indicated that MET1 is active in the male plant germinal line (Jullien *et al.*, 2010). Saze *et al.* (2003) observed that MET1 is necessary for the maintenance of the methylation patterns during plant male gametogenesis. *Arabidopsis thaliana met1* mutant pollen grains did not show faults in the vegetative cell and its function, indicating that the MET1-directed methylation does not have a essential regulatory role in the differentiation of vegetative cell (Twell, 2011). However, DNA methylation plays an important role in silencing of transposable elements in pollen (Zilberman, 2008). Recent studies have shown a relation between *de novo* DNA methylation during the male gametogenesis and siRNAs, revealing an increase in the number of transposable elements in the vegetative cell as a consequence of the lower level of DNA methylation and the absence of transcription of *MET1* and *DDM1*, chromatin remodelling factor (Slotkin *et al.*, 2009; Jullien *et al.*, 2010). According to Baroux *et al.* (2011), this would be an example in which the vegetative cell makes sacrifices to protect the genome stability of the male gametes and its transmission to the next generation.

The results presented here reveal that the microspore reprogramming to embryogenesis entails a change in the expression pattern of Bn*MET1a-like* at the transcriptional level, which highly correlates with the DNA methylation dynamics. DNA methylation and Bn*MET1a-like* expression increase highly during pollen maturation whereas they keep low levels after reprogramming. At advanced pollen embryogenesis stages, a progressive increase in Bn*MET1a-like* expression occurs, accompanying the increase in DNA methylation levels. Interestingly, the FISH assays showed a differential distribution of Bn*MET1a-like* transcripts among cell types, with a higher signal in the differentiating embryo cells. The parallelism between the FISH signals and the 5-mdC immunofluorescence intensities in different cell types during pollen embryo development suggests that the transcription of a *MET1a-like* gene and maybe a MET1-like activity could be involved, at least in part, in the increase of DNA methylation in specific cells. The results indicated an expression pattern that changes with the proliferation and differentiation processes, reaffirming the existence of an epigenetic control in the two pollen developmental pathways. Yamauchi *et al.* (2008) described a similar dynamism in the expression of *MET1* during development in stem and root cells of *Oryza sativa* L. The existence of a regulatory mechanism at transcriptional level of the genes that codify the methyltransferases in rice, together with additional mechanisms controlling the expression, has also been reported (Teerawanichpan *et al.*, 2009). Some works (Hsieh, 2000; Iida *et al.*, 2002) have indicated that low DNA methylation levels are not correlated with
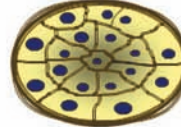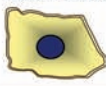
Developmental process	Stages- cell types		Global DNA methylation	*BnMET1a* expression	
Pollen gametophytic development	Vacuolated microspore		+	+	
	Mature pollen		+++	+++	Differentiated cells
Pollen embryogenic development	Pro-embryo		+	+	Proliferating cells
	Globular embryo	Inner cells	++	++	Proliferating cells
		Outer cells	+++	+++	Differentiating cells
	Cotyledonary embryo	Meristematic Cells	++	++	Proliferating cells
		Differentiating cells	+++	+++	Differentiated cells

Fig. 7. Summary of results on DNA methylation and Bn*MET1a-like* expression during gametophytic and embryogenic pollen development. +, Low; ++, medium–high; +++, highest. A common pattern of methylation level and Bn*MET1a-like* expression is seen in all differentiating cell types (grey) and all proliferating cell types (white) in both developmental processes (this figure is available in colour at *JXB* online).

low expression of *MET1*, because of the existence of active demethylating mechanisms in the cell. However, the correlation found in the current results (Fig. 7) suggests that *MET1a-like* gene could have a role not only in maintenance but also in *de novo* DNA methylation during pollen development and reprogramming, although additional methylating and demethylating mechanisms cannot be excluded.

*Zygotic and microspore embryos present analogous patterns of DNA methylation and Bn*MET1a-like *expression*

There are not many studies comparing the pattern of development of pollen and zygotic embryos; some of them have emphasized the similarities between both types of embryogenesis during the last stages of the processes (Yeung *et al.*, 1996; Ilic-Grubor *et al.*, 1998; Bueno *et al.*, 2003; Bárány *et al.*, 2005, 2010) but

epigenetic studies at the gene expression level have not been performed. The current results provide new evidence that microspore embryogenesis mimics zygotic embryogenesis, showing similar patterns of *MET1a-like* expression and DNA methylation in cotyledonary embryos formed *in vivo* and *in vitro* by the two embryogenic programmes. Proliferating cells of both pollen and zygotic embryos presented a weaker signal of 5-mdC immunofluorescence and FISH than differentiated cells. Also, RT-PCR analysis did not show significant differences of Bn*MET1a-like* expression between zygotic and microspore embryos.

Zygotic embryogenesis in plants is regulated by several important genes, and modifications in these genes could break off the embryo formation process and result in seed abortion (Huh *et al.*, 2008). Recent works (Abid *et al.*, 2011) have indicated that considerable changes in the DNA methylation pattern during embryogenesis could be involved in the disruption or maintenance of the embryogenesis process in *Phaseolus*

interspecific hybrids, supporting an earlier hypothesis that DNA methylation is critical for the regulation of plant embryogenesis gene expression. FitzGerald *et al.* (2008) suggested that MET1 is an important regulator of essential genes for the development and seed viability. Moreover, it has also been described that MET1 regulates the expression of genes involved in the cellular identity during embryogenesis, such as *YODA*, a gene involved in the specialization of the cellular embryo identity and suspensor formation (Lukowitz *et al.*, 2004). In fact, *met1* mutants show altered cellular divisions in the suspensor part as well as in the embryo proper cells from the early embryogenesis stages (Xiao *et al.*, 2006).

These data suggest the participation of *MET1a-like* genes in the regulation of the cellular differentiation that takes place at the advanced stages of zygotic and pollen embryo development, show a similar expression pattern for Bn*MET1a-like* genes in the two embryogenic programmes, and provide support for similar epigenetic changes in both developmental pathways.

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