Nuclear bodies domain changes with microspore reprogramming to embryogenesis

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We analysed the presence of nuclear bodies and particularly Cajal bodies during representative stages of gametophytic and haploid embryogenic development in isolated microspore and anther cultures of a model system (*Brassica napus cv.* Topas) and a recalcitrant species (*Capsicum annuum L.* var. Yolo Wonder B). The nuclear bodies domain is involved on several important roles on nuclear metabolism, and Cajal bodies are specifically involved on the storage and maturation of both snRNPs and snoRNPs, as well as other splicing factors, necessary for mRNA and pre-rRNA processing, but not directly on the transcription. In this study, immunofluorescence and immunogold labelling with anti-trimethylguanosine antibodies against the specific cap of snRNAs, ultrastructural and cytochemical analysis were performed on cryoprocessed samples at confocal and electron microscopy respectively. Results showed that Cajal bodies increase during the early stages of microspore embryogenic development (young pro-embryos), compared to microspore and pollen development. Our results suggest that Cajal bodies may have a role in the transcriptionally active, proliferative stages that characterise early microspore embryogenic development.

Key words: microspore embryogenesis, nuclear architecture, nuclear bodies, Cajal bodies, *Brassica napus, Capsicum annuum*, confocal microscopy, transmission electron microscopy.

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Microspore embryogenesis is defined as the deviation of the gametophytic pathway towards embryogenic development and haploid or doubled haploid plant regeneration. This change in developmental program is generally induced by a pre-treatment with mild physical and/or chemical stress (heat, cold, starvation, etc; Chupeau *et al.,* 1998; Maluszynski *et al.,* 2003). Not all the cells present in the culture are equally sensitive to the pre-treatment, some of them are effectively induced (deviated/reprogrammed) microspores but others are non-induced microspores that continue the gametophytic development (Custers *et al.,* 1994; Seguí-Simarro, 2001). Therefore, it is important to identify cellular markers of the embryogenic developmental program. Synthesis of heat shock proteins (hsps) and mitogen-activated protein kinases (MAPKs), as well as rearrangements in the cell cytoskeleton have been previously proposed as cellular markers of this change (Zarsky *et al.,* 1995; Binarova *et al.,* 1997; Simmonds and Keller, 1999; Bárány *et al.,* 2001; Pechan and Smykal, 2001; Coronado *et al.,* 2002; Seguí-Simarro *et al.,* 2003, 2005). In addition, defined nuclear domains seem to be the targets for the nuclear translocation of hsps and MAPKs. Specific domains of the interchromatin region, previously identified as transcription sites (Testillano *et al.,* 1993) are enriched in MAPKs and Hsp70 (Bárány *et al.,* 2001; 2005; Coronado *et al.,* 2002; Seguí-Simarro *et al.,* 2003, 2005).

Several structural evidences indicate that the nucleus is one of the cell compartments that change its functional organization as a consequence of microspore embryogenesis induction (Testillano *et al.,* 2000; 2005). In many different plant and animal cell types, it has been established a direct relationship between the functional organization of the nucleus and the metabolic status of

the cell (Fakan and Puvion, 1980; Risueño and Medina, 1986; Raska *et al.,* 1991; Andrade *et al.,* 1993; Spector, 1993; González-Melendi *et al.,* 1998; Seguí-Simarro, 2001, Fakan, 2004). Within the nucleus, the nucleolus and the interchromatin region are nuclear domains that remodel their architecture after induction (Risueño and Testillano, 1994; Testillano *et al.,* 2005). Nuclear bodies have recently been proposed as potentially sensitive to changes in proliferative activity in plant cells (Testillano *et al.,* 2005).

Cajal bodies (CBs) were firstly described in neurons by Ramón y Cajal (1903) as *accessory bodies* because of their frequent location near the nucleolus and their similar silver staining. Due to their convoluted architecture, they were named *coiled bodies* by Monneron and Bernhard (1969) in mammalian cells and afterwards in plants by Moreno-Díaz de la Espina and coworkers (1982b). CBs are present as constitutive nuclear elements of both differentiated and proliferating animal (Gall, 2000) and plant cells (Risueño and Medina, 1986; Beven *et al.,* 1995; Straatman and Schel, 2001). Structurally, CBs are round-shaped domains of up to 2.0 μm, appearing in electron micrographs as packed, coiled thick threads embedded in the interchromatin region (Beven *et al.,* 1995; Lafarga *et al.,* 1998; Fakan, 2004; Raska *et al.,* 2005). They have been frequently found associated with the nucleolus and related or connected to the interchromatin granules (Risueño and Medina 1986). CBs are characterized by the presence of the p80 coilin protein, the Sm antigen and several small nuclear RNAs (snRNAs), as well as the nucleolar proteins fibrillarin, Nopp140 and the S6 ribosomal protein (revision by Gall, 2000). Functionally, CBs are not well understood. It seems clear that they are not directly engaged in mRNA splicing, (Huang *et al.,* 1994), but in the maturation of both snRNPs and snoRNPs, in the processing of histone mRNA 3′ ends (Dominski and Marzluff, 1999) and biogenesis and storage of transcriptosome and splicing factors (Spector, 1993; Gall, 2000; Proudfoot, 2000), snRNPs (Carvalho *et al.,* 1999) and snoRNPs (Narayanan *et al.,* 1999; Platani *et al.,* 2000).

Brassica napus is considered a model species for the study of microspore embryogenesis (Chupeau *et al.,* 1998), but the cellular mechanisms controlling the process are still not well known. In contrast, some economically interesting crops, as *Capsicum annuum*, have been considered recalcitrant species, due to the extreme difficulty to deviate the microspore towards embryogenesis. However, in the last years several groups have successfully obtained haploid embryogenic regenerants in pepper (Mitykó *et al.,* 1995; Bárány *et al.,* 2001, 2005). In this study, we aimed to study the changes in nuclear bodies, specially focusing on CBs, during the switch to microspore embryogenesis in both a model (*Brassica napus*) and a recalcitrant (*Capsicum annuum*) species, in order to evaluate their potential use as markers of the change in developmental program. Our results show that NBs and specifically CBs increase their number after microspore embryogenesis induction in reprogrammed pro-embryo cells, which are in proliferation, compared to the differentiating cell stage of the microspores and pollen that follow the gametophytic programme.

Materials and Methods

Plant material

Brassica napus L. Cv. Topas and *Capsicum annuum* var Yolo Wonder B donor plants were grown as previously described (Seguí-Simarro *et al.,* 2003; Bárány *et al.,* 2005). Brassica microspore culture and embryogenesis induction (16 h. at 32.5°C) was performed according to (Jouannic *et al.,* 2001). *Capsicum* anthers were excised and cultured as described (Bárány *et al.,* 2005).

Antibodies

Anti-2,2,7 trimethyl guanosine (TMG) from Calbiochem (Clone K121) was used for Cajal bodies identification by immunofluorescence and TEM immunogold labelling. The antibody cross-reacts with the specific cap of the small nuclear RNAs (snRNA) present in the small nuclear ribonucleoproteins (snRNPs) of the mRNA splicing machinery.

Immunofluorescence

Microspore and haploid embryo cultures were processed for cryomicrotomy and cryosectioned as previously described (Seguí-Simarro *et al.,* 2003). Semithin (1 μm) cryosections were obtained and placed on multiwell glass slides. After thawing the sections, immunofluorescence was performed as described (Seguí-Simarro *et al.,* 2003), using anti-

TMG as the primary antibody diluted 1/100 and incubated for 1 hour at room temperature in darkness, and secondary antibody (anti-mouse IgG-Cy3) diluted 1:25 in 1% BSA in PBS, and incubated for 45 minutes in darkness. Sections were stained with DAPI prior to observation in a Bio-Rad MRC-100 confocal scanning head coupled to a Zeiss Axiovert 135 microscope. Controls were performed excluding the primary antibody from the incubation buffer.

Electron microscopy and cytochemistry

Samples to be observed for electron microscopy were fixed in Karnovsky fixative (4% formaldehyde + 5% glutaraldehyde in 0.025M cacodilate buffer, pH 6.7), post-fixed in 2% OsO4, dehydrated in an ethanol series for 3 days and slowly embedded in Epon resin for 2 days. Epon blocks were polymerized at 60°C for 2 days. ~80 nm thin sections were collected on 75-mesh copper grids, counterstained with uranyl acetate and lead citrate and observed in a JEOL 1010 TEM operating at 80 kV. Several samples were fixed in 4% formaldehyde in PBS and dehydrated by the PLT (Progressive Lowering of Temperature) method in a Leica AFS system. Then, samples were infiltrated and polymerized at -30°C in Lowicryl K4M resin under UV light. Some ultrathin sections were stained by the EDTA regressive staining of Bernhard (1969), a preferential cytochemical method for RNPs.

Immunogold labeling

Microspores and haploid embryos at different stages were prefixed in formaldehyde, cryoprotected in 2.3M sucrose, cryofixed in liquid nitrogen and cryo-processed as described (Seguí-Simarro *et al.,* 2003). Samples were freeze-substituted in methanol + 0.5% uranyl acetate at -80°C for 3 days, infiltrated in Lowicryl K4M and polymerized at -30°C under u.v. irradiation in a Leica AFS system. Thin (80 nm) sections were placed on nickel grids. The grids were sequentially floated in PBS, 5% BSA in PBS, and anti-TMG diluted 1/100, 1 hour at room temperature. After several washes in 1% BSA in PBS, the grids were incubated with anti-mouse IgG-gold, 10 nm; (Biocell, Cardiff, UK) diluted 1:25 in 1% BSA, for 45 minutes at room temperature, washed, air dried, counterstained and observed in a JEOL 1010 TEM at 80 kV. Controls were performed excluding the primary antibody.

Results

Cellular rearrangements after microspore reprogramming to embryogenesis

Brassica napus microspore cultures and *Capsicum annuum* anther cultures were used to study the changes in the nuclear bodies domain. First we comparatively checked the progress of both *in vitro* systems in comparison with the *in vivo* development. During the studied stages of gametophytic development and the first stages of microspore embryogenic development, both systems behaved similarly in terms of developmental stages and patterns. Donor anthers of both *Brassica napus* and *Capsicum annuum* containing microspores at the stage of late vacuolate microspores were selected (Figure 1A, A′, B, B′). They mostly contained vacuolate microspores which are characterised by the presence of a large central vacuole that displaces the nucleus to an off-centred position. In *Brassica*, the chromatin pattern, as revealed by DAPI staining with faint fluorescence signal, was essentially decondensed, with few, small bright foci indicating condensed chromatin masses, generally at the periphery of the nucleus (Figure 1A′, C′). In *Capsicum*, chromatin fluorescence after DAPI staining appeared as a strong and quite homogeneous signal, indicating a compacted chromatin pattern (Figure 1B′, D′). After the first pollen mitosis, two different cells form the young pollen grain, the vegetative and the generative. As the pollen grain matures, the generative cell migrates towards the centre of the cell, as evidenced by the central disposition of the generative cell which is quite close of the vegetative nucleus (Figure 1C, C′, D, D′). The cromatin of the generative nucleus appears in a highly condensed state, as evidenced by the brightness of DAPI staining, compared with the faint fluorescence of the vegetative nucleus. In *Brassica*, the second pollen mitosis takes place in the pollen grain, giving rise to the tricellular mature pollen, in contrast to the bicellular stage of the mature pollen in *Capsicum*.

In those microspores successfully induced to embryogenesis, successive cell divisions lead to structurally similar cells, instead of the morphologically different generative and vegetative cells of the pollen grain. Young pro-embryos are composed of multicellular, indiferentiated cells that actively proliferate within the microspore exine wall until

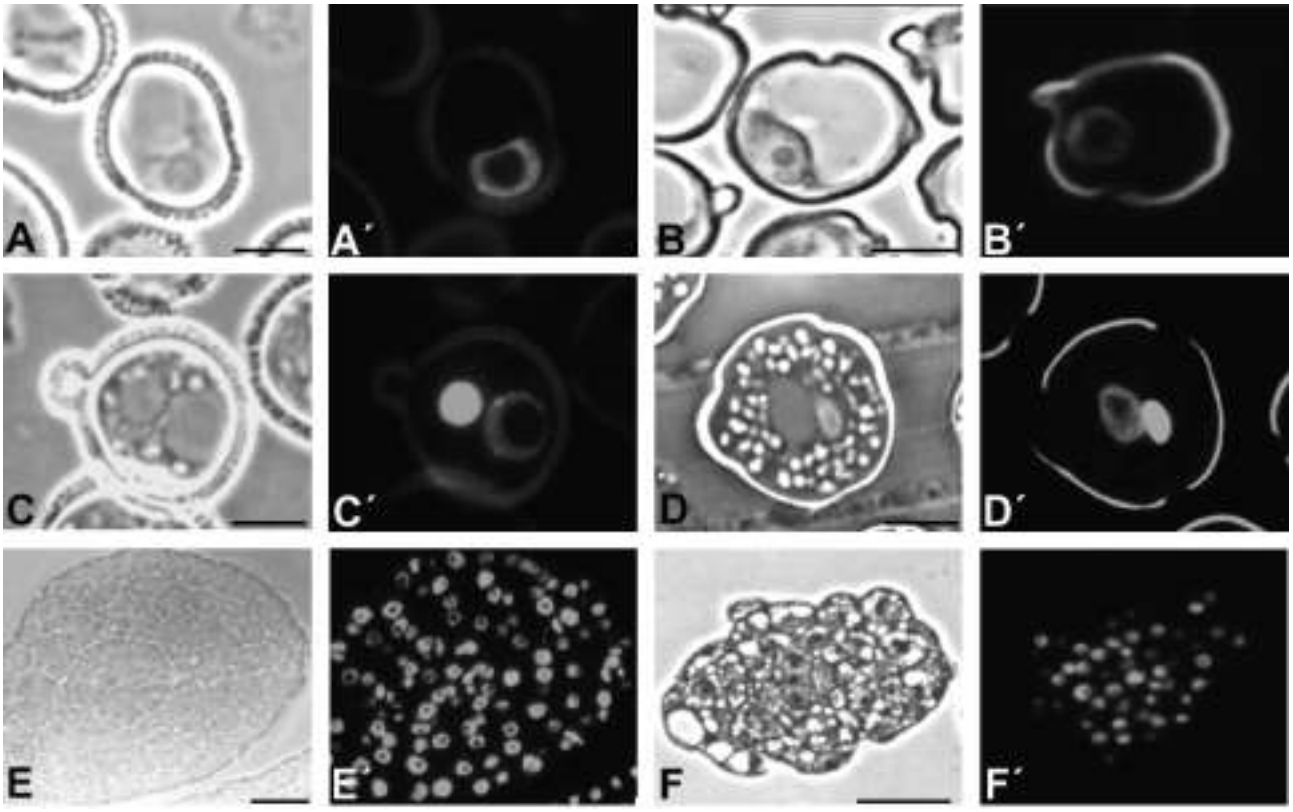

Figure 1. ***Brassica napus*** **(A, C and E pairs) and** ***Capsicum annuum*** **(B, D and F pairs) stages of gametophytic and induced microspore embryogenic development. A-F. Phase contrast images. A'-F'. DAPI-staining images. A-B'. Vacuolate microspores. C-D'. Bicellular pollen. E-F'. Young pro-embryos after the burst of the exine. Bars in A-D': 10 µm. Bars in E-F': 50 µm.**

they burst out (Figure 1E, E′, F, F′). Both *Brassica napus* and *Capsicum annuum* proembryo cell nuclei display a faint DAPI fluorescence spread throughout the nucleoplasm.

The nuclear bodies domain during pollen developmental pathways

The nuclei of *Brassica* microspores and proembryos (Figure 1 A′, E′) had a large interchromatin region, a generally centred nucleolus and small masses of condensed chromatin which were frequently abutted to the nuclear envelope. In *Capsicum*, chromatin pattern was more condensed, with small and numerous patches connected by chromatin threads of different thickness (Figures 2A-C, 3A, 5A). Also, a conspicuous nucleolus can be observed. In addition, nuclear bodies (NBs) are located in the interchromatin region as constitutive elements of the nuclear architecture (Figures 2A, B, 3, 4, 5A-B); they were frequently observed in the same section plane of the nucleolus and associated with it (Figures 2B, 3C, 4C, 5B). Among the NBs, one of the most distinct bodies are the Cajal bodies (CBs). They have been described to be anti-TMG positive (Testillano *et al.,* 2005). In this study, we used this antibody to specifically identify CBs among the rest of NBs (Figures 2D, 5D-E) in order to analyze the Cajal bodies domain during pollen development and the changes that accompany the reprogrammed microspore embryogenesis.

During *Capsicum* and *Brassica* microspore development, NBs were present in the interchromatin region (Figures 2A, 3A, 4A), also in the same section plane of the nucleolus and frequently in close association with the nucleolar components (Figures 3C, 4C), but not in relation to the condensed chromatin patches. After the first pollen division, young bicellular pollen exhibited a similar presence of NBs, always present in the highly active vegetative nucleus (Figure 2B). Conversely, in mature pollen NBs were very scarce, being most of the observed nuclei devoid of them (Figure 2C). Anti-TMG immunofluorescence pattern consisted of a faint, diffuse signal spread through the nucleoplasm (Figure 2D, F). Additionally, in some microspores (Fig 2D) and young bicellular pollen (data not

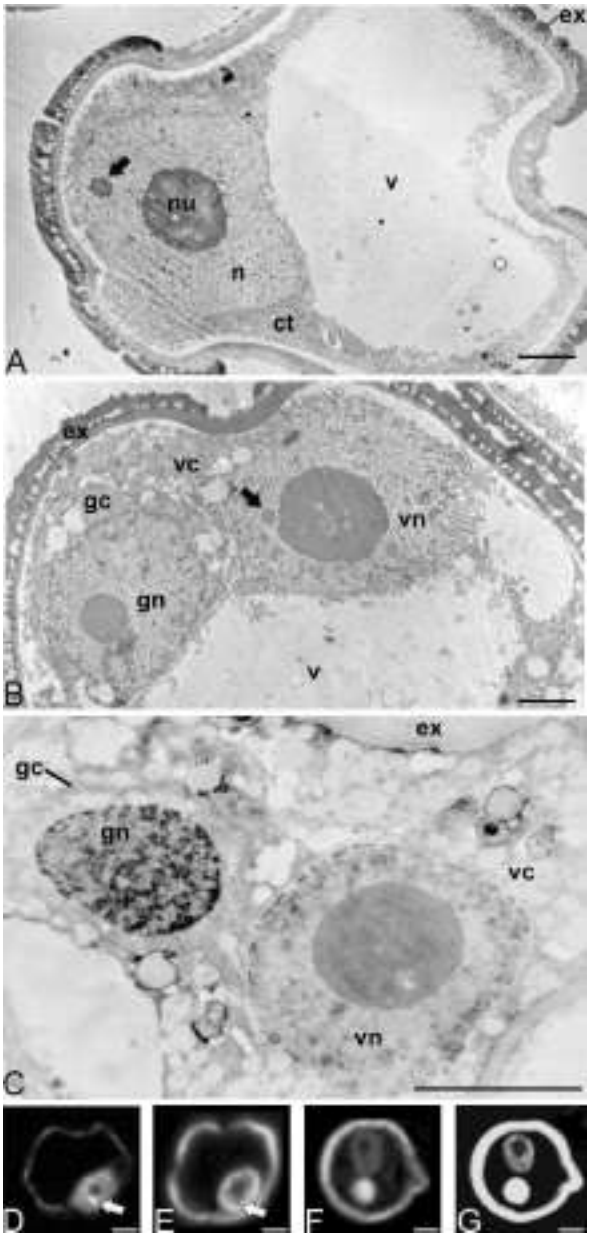

Figure 2. Nuclear bodies and chromatin organization pattern during gametophytic development. A-C. Electron micrographs. A. Vacuolate microspore. B. Young bicellular pollen grain. The black arrow points to nuclear bodies appearing in the same plane of the nucleolus, and frequently associated with it. Different chromatin condensation degrees are shown: A, decondensed pattern; B, C. Decondensed in vegetative nuclei, and condensed in generative nuclei. D-G. Anti-TMG immunofluorescence (D, F) and DAPI-staining (E, G) of a vacuolate microspore (D, E) and a mature bicellular pollen grain (F, G), white arrows point to a CB (D) and to the absence of fluorescence in the corresponding regions of the DAPI-stained image (E). ct. Cytoplasm; ex. Exine; gc. Generative cell; gn. Generative nucleus; n. Nucleus; nu. Nucleolus; v: Vacuole; vc. Vegetative cell; vn. Vegetative nucleus. Bars in A-C: 2 µm. Bars in D-G: 5 µm.

shown) a bright spot emerged over the diffuse signal. DAPI staining of the same sections (Figure 2E) revealed that the bright spots do not overlap with the DAPI-stained DNA, confirming the absence of DNA in these bodies. We could never detect more than one per section of developing microspore/pollen nucleus (Figures 2A, B, 3A, 4A). In mature pollen grains, both the observed generative and vegetative nucleus showed no bright spots after anti-trimethyl guanosine immunofluorescence (Figure 2F, G).

The electron microscopy analysis of the CBs present in the microspore revealed that they were rounded bodies with a fine structure showing dense coiled threads immersed in a low dense material (Figures 3 and 4). The EDTA regressive staining for RNPs preferentially contrasted, in the microspore, the nucleolus and interchromatin structures, including the CBs (Figure 4), also revealing that they were in connection with fibrillo-granular structures of the interchromatin region (Figure 4B, C). Interchromatin granules could also be seen in connection with the CBs (Figures 3C, 4C).

Changes in the nuclear bodies domain after the switch to embryogenesis

In contrast to the pollen developmental programme, during induced microspore embryogenesis, NBs were consistently found in embryogenic microspores symmetrically divided and developing pro-embryos (Figure 5). NBs in TEM micrographs (Figure 5A) appeared as dense, round-shaped masses of variable size, with a thick, coiled fibrillar texture, similar to those in proliferating cells of root meristems (Moreno-Díaz de la Espina *et al.,* 1982a, 1982b). In some sections, up to two NBs were observed in the same section (arrows in Figure 5B), and were frequently found apposed to the nucleolar surface. CBs appeared also connected with the interchromatin granules (Figure 5B), as it has been reported in root meristems (Moreno-Díaz de la Espina *et al.,* 1982a, b). Anti-TMG immunofluorescence labelling (Figure 5D) and DAPI staining (Figure 5C) of young pro-embryos revealed the presence of at least one CB, in most of the cells present in the same pro-embryo section, often close to the nucleolus. The bright spots of anti-TMG fluorescence (arrows in Figure 5D) coincided with a dark spot when imaging the DAPI staining of the same cells (arrows in Figure 5C), indicating that bright spots, likely CBs, locate in the interchromatin region. At the electron microscopy level, anti-TMG immunogold labelling of pro-embryo cells revealed the presence of some gold particles dispersed

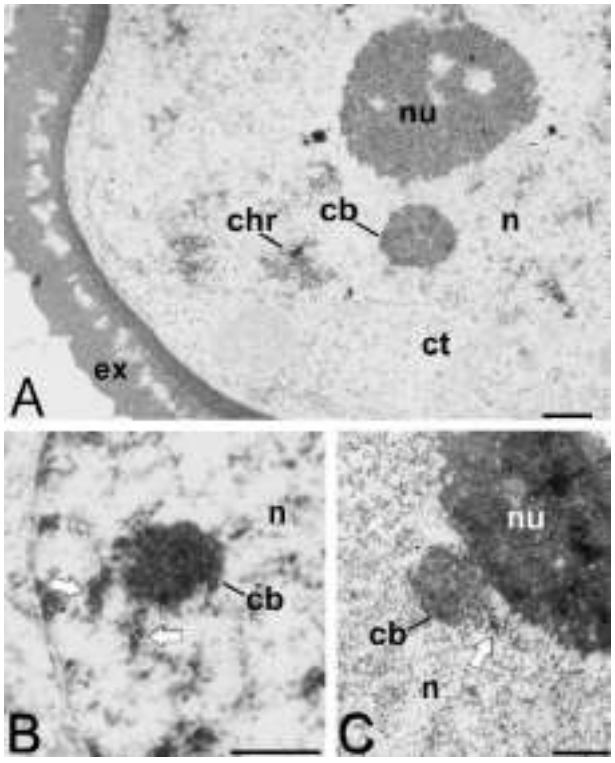

Figure 3. Ultrastructural organization of Cajal bodies in microspore nuclei. A. Capsicum microspore nucleus with a Cajal body (cb) in the interchromatin region. B, C. High magnification micrographs of CBs in the interchromatin region (B) and associated with the nucleolus (C), showing a coiled structure and connections with interchromatin structures (white arrows). n: nucleus, nu: nucleolus, chr: chromatin, ct: cytoplasm, ex: exine. Bars represent 250 nm.

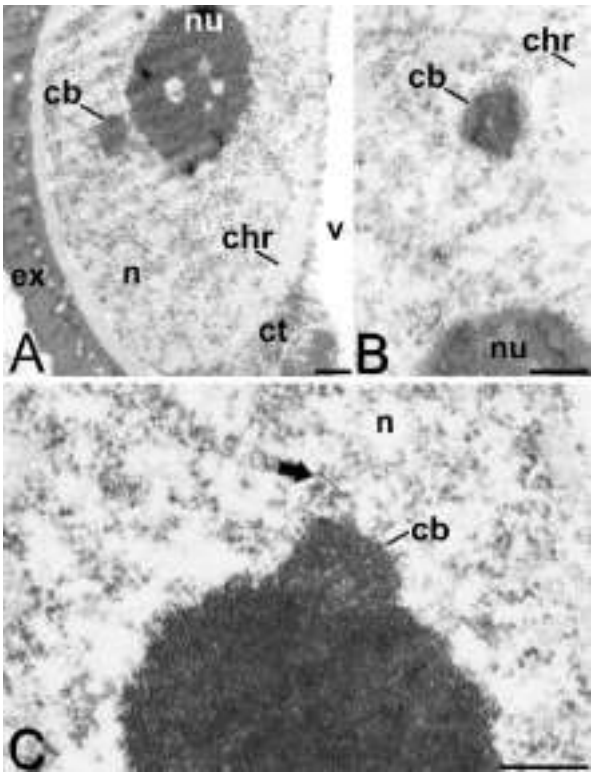

Figure 4. Fine structure of Cajal bodies as revealed by EDTA cytochemistry for RNPs. A. Capsicum microspore nucleus with the condensed chromatin (chr) bleached by the EDTA; the interchromatin structures, the nucleolus (nu) and Cajal body (cb) appeared preferentially contrasted. B, C. High magnification micrographs of CBs in the interchromatin region (B) and associated with the nucleolus (C); EDTA-positive fibrillar structures (arrow) are observed connecting the CB with interchromatin structures. n. Nucleus, ct. Cytoplasm, ex. Exine. Bars: 250 nm.

through the interchromatin region (Figure 5E) corresponding to the snRNPs, but densely accumulated specifically over some NBs, with a size, shape, ultrastructure and nuclear location similar to the CBs described in several plant systems under different developmental conditions. This pattern of labelling was very specific, as demonstrated by control assays excluding the first antibody (data not shown). In young pro-embryos, two or three NBs could be also observed in a single cell section in some occasions (Figure 5B). Anti-TMG-positive CBs seem to increase in microspore-derived young pro-embryos (Figures 5C-E).

Discussion

Brassica napus and *Capsicum annuum* are representative examples of a model, easily inducible and a recalcitrant species, in terms of response to stress treatments switching the microspore to embryogenesis. Both systems have several common characteristics, such as the establishment of the vacuolate microspore as the most responsive stage for microspore embryogenesis induction and the need for a heat stress as inductive treatment (Seguí-Simarro *et al.,* 2003; Bárány *et al.,* 2005), although the efficiency in response to such treatment greatly differs between them (Custers *et al.,* 1994; Mitykó *et al.,* 1995).

The comparison of both systems in terms of gametophytic and induced microspore embryogenic development showed a remarkable similarity (Figure 1), as expected. The parallel analysis in both systems of the changes in the chromatin pattern and in the NB domain showed no differences between the two species during microspore and pollen development, and during the switch to embryogenesis. Interphase chromatin is organized in irregular patches which occupy specific territories in the

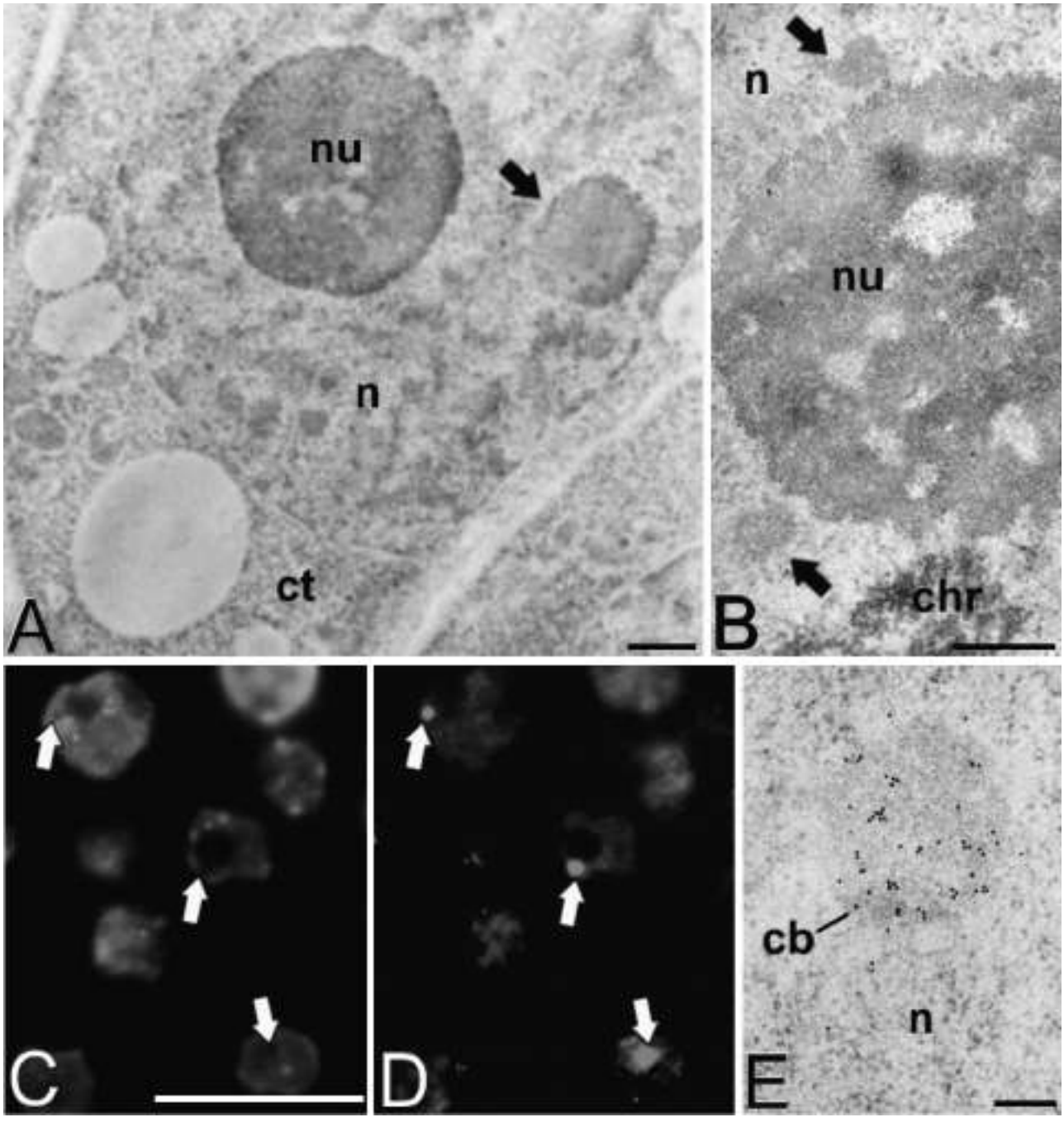

Figure 5. Nuclear bodies during induced microspore embryogenic development. A-B. TEM micrographs of a *Capsicum* (A) and *Brassica* (B) pro-embryo cells, showing NBs of different sizes (black arrows). C-D. DAPI staining (C) and anti-TMG immunofluorescence (D) of a young *Brassica* pro-embryo, showing intensely stained CBs (white arrows in D), and the absence of fluorescence in the corresponding regions of the DAPI-stained image (white arrows in C). E. Electron micrograph of anti-TMG immunogold labeling of a young Brassica pro-embryo cell, showing the accumulation of gold particles over a CB. cb. Cajal body; chr. Condensed chromatin; ct. Cytoplasm; n. Nucleus; nu: Nucleolus. Bars in A, B, E: 500 nm. Bars in C-D: 10 µm.

nucleus. It has been reported that the interior of the nucleus houses gene-rich chromosomes while gene-poor chromosomes are at the periphery. Thus, non-transcribed sequences are located in the nuclear periphery while active genes and gene-rich regions tended to localize on regions exposed inwards or in loops extending from the territories (Dietzel *et al.,* 2004; Gorisch *et al.,* 2005). This way, the rearrangement of the chromatin pattern and location observed during the different stages of microspore embryogenesis would reflect the specific requirements of the developmental pathway.

These requisites are: an increase of activity during the proliferative stages and a decrease of activity during differentiation, as occurs during microspore gametophytic development.

The biological activities of the nuclear bodies as well as their localization and dynamics seem to be closely related to gene expression. Cajal bodies and PML bodies translocate to specific regions within the nucleus, apparently related to the dynamic organization and accessibility of the chromatin, since properties of the chromatin environment determine the mobility of Cajal (and PML) bodies (Gorish *et al.,* 2005). In proliferating plant cells, it has been reported that CBs can even translocate from the nucleus to the cytoplasm (Moreno-Díaz de la Espina *et al.,*. 1982a, Risueño and Medina 1986).

CBs are not involved on mRNA splicing, since poly (A) RNA and nascent mRNA transcripts have never been observed within them (Moreno-Díaz de la Espina *et al.,* 1982a, b; Huang *et al.,* 1994; Straatman and Schel, 2001). In plants, it has been suggested that CBs are involved on rRNA processing, transport and storage, but not in transcription (Moreno Díaz de la Espina *et al.,* 1982a, b, Risueño *et al.,* 1978). It seems that CBs are nuclear storage sites where the transcriptosome and splicing factors mature prior to recruitment to transcription and splicing sites (Spector, 1993; Gall, 2000; Proudfoot, 2000). Despite this fact, they have been related to transcriptional activation, cell cycle progression and metabolic activity in mammalian cells (Spector, 1993; Pena *et al.,* 2001; Fernandez *et al.,* 2002; Fakan, 2004; Testillano *et al.,* 2005). In *Brassica* and *Capsicum*, CBs were present in vacuolate microspores and the vegetative cell of young pollen grains, just after the first pollen mitosis, but in a limited number. These stages have been characterised as biosynthetically active (Risueño *et al.,* 1988; Testillano and Risueño, 1988), with high transcriptional activity (Mascarenhas, 1989). However, in mature pollen grains, with the lowly active vegetative cell arrested in G0, presence of NBs is rarely observed, suggesting a correlation between transcriptional activity and presence of CBs. During microspore embryogenic development, young pro-embryos are highly engaged in proliferation (Seguí-Simarro *et al.,* 2005). In pro-embryo cells, the frequency of CB-containing cells and the number of CBs per cell increased. Therefore, our results favour the notion that the number of CBs increase in response to an increase in cell proliferation activity (Andrade *et al.,* 1993; Fernandez *et al.,* 2002), which conceivably must be reflected in a higher transcription and processing of the nascent transcripts.

The relationship between CBs and the nucleolus could also be related with the entry into proliferation. Numerous studies in animal and plant cells, including the present one, have evidenced a frequent association between these two structures (Moreno Díaz de la Espina *et al.,* 1982a, b, Risueño and Medina, 1986; Narayanan *et al.,* 1999; Platani *et al.,* 2000; Gall 2000; Segui-Simarro 2001; Testillano *et al.,* 2005). Studies on mammalian live cells have shown that CBs move to and from nucleoli (Platani *et al.,* 2000). The association of CBs with the nucleolus has been related to a role for CBs in the biogenesis pathway and transport of snoRNPs involved on pre-rRNA processing (Narayanan *et al.,* 1999; Platani *et al.,* 2000). The frequent association observed between CBs and the nucleolus in developing microspores and proliferative pro-embryos would imply an increased nucleolar recruitment of rRNA processing machinery components, as a reflection of an increased nucleolar activity during proliferation, where a massive synthesis of ribosomes is needed. Since the activation of the proliferative activity is associated to the induction of embryogenesis mediated by stress, CBs could also be related to the entry into proliferation by the response to the inductive treatment and, maybe, would also be involved indirectly in the microspore embryogenesis progress.

In summary, nuclear bodies and in particular Cajal bodies increase their presence in transcriptionally active, proliferative cells during microspore embryogenic development in *Brassica* and *Capsicum*, defining them as potential cell markers of the entry into proliferation due to the reprogramming of the developmental pathway to the embryogenesis.

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