MICROSPORE-DERIVED EMBRYOGENESIS IN *Capsicum annuum* L.: SUBCELLULAR REARRANGEMENTS THROUGH DEVELOPMENT

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ABSTRACT

Background: *In vitro* cultured microspores, after an appropriate stress treatment, can switch towards an embryogenic pathway. This process, known as microspore embryogenesis, is an important tool in plant breeding. Basic studies on the process in economically interesting crops, especially in recalcitrant plants, are very limited and the sequence of events is poorly understood. *In situ* studies are very convenient for an appropriate dissection of the microspore embryogenesis, a process in which a mixture of different cell populations (induced and non-induced) develop asynchronically.

Results: In this work, the occurrence of defined subcellular rearrangements has been investigated during early microspore embryogenesis in pepper, an horticultural crop of agronomic interest, in relation to proliferation and differentiation events. Haploid plants of *Capsicum annuum* L. var. Yolo Wonder B have been regenerated from *in vitro* anther cultures by a heat treatment of 35ºC for 8 days. Morphogenesis of microspore-derived embryos has been analyzed at both light and electron microscopy levels, using low temperature processed, well-preserved specimens. The comparison with the normal gametophytic development revealed changes in cell organization after embryogenesis induction and permitted the characterization of the time sequence of a set of structural events, not previously defined in pepper, related to the activation of proliferative activity and differentiation. These changes mainly affected the plastids, the vacuolar compartment, the cell wall and the nucleus. Further differentiation process mimicked that of the zygotic development.

Conclusions: The reported changes can be considered as markers of the microspore embryogenesis. They increased the understanding of the mechanisms controlling the switch

and progression of the microspore embryogenesis, which could help to improve its efficiency and to direct strategies, especially in agronomically interesting crops.

INTRODUCTION

Microspores can switch their normal gametophytic development *in vitro* towards an embryogenic pathway. The process, known as microspore embryogenesis, represents an important tool in plant breeding since it allows the generation of isogenic lines and new varieties through double-haploid plants (Chupeau *et al.* 1998). Microspore-derived embryogenesis is highly dependent on the species, genotype, physiological state of the donor plants, induction conditions, culture media composition and state of development of the explants (Guha and Maheshwari 1964; Guha-Mukherjee 1999; Heberle-Bors 1985; Raghavan 1986; Reynolds 1997; Vicente *et al.* 1991; Custer *et al.* 1994, Kuhmlen and Lorz, 1999, Touraev *et al* 1997). The most widely used method to induce microspore embryogenesis in several species that are particularly recalcitrant for plant breeding purposes, is anther culture. It has been applied to many species, including crops and trees (Bueno *et al.* 1997; Germanà *et al.* 1991). In the *Solanaceae* family, this process has been triggered in potato (Teparkum and Veilleux 1998, Rokka *et al.* 1996, Chani *et al.* 2000, Boluarte-Medina and Veilleux 2002), tomato (Zagorska *et al.* 1998, 2004, Shtereva *et al.* 1998, Ziv *et al.* 1984, Zamir *et al.* 1980) and pepper (Gyulai *et al.* 2000, Dolcet-Sanjuan *et al.* 1997, Sibi *et al.* 1979, Mitykó *et al.* 1995, Bárány *et al.* 2001), among others, with variable efficiency. *Capsicum annuum* L., is an economically interesting crop in horticulture and several protocols have been reported to induce microspore embryogenesis and plant regeneration in different varieties (Dumas de Vaulx *et al.* 1981, Mitykó *et al.* 1995, Dolcet-Sanjuan *et al.* 1997, Bárány *et al.* 2001).

Basic studies on the process have been mainly pursued in model species like rapeseed, tobacco and barley (Chupeau *et al.* 1998) but reports on other economically interesting crops are very limited and the sequence of events is poorly understood. In comparison with the

molecular analysis, *in situ* studies provide data on the structural organization and the expression and subcellular localization of molecular targets in individual cells with defined structural features that are informative as to their developmental stage. Cellular studies are very convenient for an appropriate dissection of the microspore embryogenesis, a process in which a mixture of different cell populations (induced and non-induced) develop asynchronically. The cellular events taking place in the first stages after embryogenic induction are necessary data to increase the understanding of the mechanisms controlling the switch and progression of the embryogenesis pathway; this would help to improve the efficiency of the process and to direct strategies, especially in agronomically interesting crops. The search of molecular and cellular markers during early stages of microspore embryogenesis has been pursued to monitor the physiological processes involved in the induction, to identify the cells committed to the new programme and the correct development of embryos and plants. Nevertheless, cellular studies analyzing sequential structural events on microspore embryogenesis are not abundant and frequently directed to model species (Binarova *et al.* 1993, Hause and Hause 1996, Reynolds 1997). Recent works have reported cellular changes accompanying early stages of the process and their relation to cell developmental fate (Coronado *et al.* 2002, Ramírez *et al.* 2004, Testillano *et al.* 2005). The development of plastids and starch accumulation constitutes a differential feature during pollen formation in many species (Franchi *et al* 1996), as well as in defined stages of zygotic embryogenesis (Raghavan 2000). An uneven starch accumulation has been observed and suggested to be associated with polarity establishment, cell differentiation and fate (Indrianto *et al.* 2001, Testillano *et al.* 2000, Raghavan 2000).

A few previous works have reported some cellular features of microspore embryogenesis in *Capsicum annuum* (González-Melendi *et al* 1995, 1996, Testillano *et al.* 1995, Bárány *et*

al. 2001, Kim *et al.* 2004), but a detailed ultrastructural characterization of sequential events of microspore-derived structures was still lacking, until the present work. In this paper, the subcellular rearrangements at the early development of microspore embryogenesis in *Capsicum annum* have been analyzed in comparison with the normal gametophytic development, with special attention to the changes affecting nuclei, cell walls, plastids (starch) and vacuoles. In this work, previous protocols for *in vitro* anther culture in *Capsicum* (Dumas de Vaulx *et al.* 1981), later slightly modified by Mitykó *et al.* (1995) have been applied to induce the process. The cellular characterization of the progression of haploid embryogenesis has been pursued at both light and electron microscopy levels using low-temperature processed, well preserved specimens. The results revealed the occurrence of defined subcellular rearrangements and permitted the characterization of sequential developmental stages. The reported changes can be considered as markers of the embryogenesis process, being modulated in relation to proliferation and differentiation events.

RESULTS

Process of embryogenesis by anther culture

In order to identify and select the starting material at the onset of the culture, an initial analyses of the flower bud morphology, the size of the anthers and the developmental stage of the microspores were performed by DAPI staining on squashes. Anthers from buds with a similar length of sepals and petals (Figure 1a) were the most responsive for embryogenesis induction. These preferentially contained late vacuolated microspores (Figure 3a-3d), as determined by DAPI staining.

After six days in culture, responsive anthers swelled and increased in size up to 1.5 ± 0.2 fold (Figure 1b). Many other anthers became dark and lost turgescentness. Twenty to thirty days later white, elongated structures emerged from the responsive anthers (Figure 1c). These were identified as embryos, showing roots and shoots at later stages (Figure 1d). These embryos proceeded through the cotyledonal stage (Figure 1e) and finally towards green plantlets with leaves (Figures 1f, g). After 80 days, the regenerated plants developed a normal anatomy but, in some cases, they were smaller, with less leaves (Figure 1h, left side) and grew less vigorously than control diploid plants (Figure 1h, right side).

Flow cytometer analysis of a propidium iodide-stained population of nuclei from leaves of regenerated plants determined that both haploids (Figure 2a) and doubled haploids (Figure 2b) were obtained, as compared to a diploid control profile (Figure 2c). The diploid profile showed two peaks (DNA ratio 1:2), in all cases the highest peak corresponded to G1, and the smallest to G2. When material from the haploid and the diploid samples was mixed and

measured (Figure 2d), two G1 peaks were clearly resolved, indicating that they correspond to 1C and 2C values. Small regenerated plants (Figure 1h, left side) always showed a haploid profile (Figure 2a).

Normal gametophytic development

The cellular organization during the normal gametophytic development was analyzed in order to compare it with *in vitro* embryogenesis. Anthers of increasing sizes were excised from plants and analyzed at both light and electron microscopy levels.

Vacuolated microspores exhibited a large vacuole which pushed the nucleus to the cell periphery (Figures 3a, 3b). No starch was detected by the Iodide-based staining at this stage (Figure 3c). At the ultrastructural level, (Figure 3d) the nucleus displayed a decondensed chromatin pattern with small patches, abundant fibrillo-granular interchromatin structures and a large nucleolus with an abundant granular component; this resembled a typical organization of a nucleus with high transcriptional activity. The cytoplasm formed a thin layer below the intine (Figure 3d).

After the first mitosis, the young bicellular pollen grain was formed (Figures 3e-3h). The generative cell appeared attached to the exine and in close vicinity with the vegetative nucleus, which was surrounded by large vacuoles that were still present (Figures 3e, 3h). DAPI staining revealed different fluorescent intensities in the generative and vegetative nuclei (Figure 3f) corresponding to different degrees of chromatin condensation in both nuclei. The generative nucleus exhibited condensed chromatin and compact nucleolus, whereas the vegetative one displayed a more decondensed chromatin (Figures 3f, 3h). As pollen

maturation proceeded, in mid bicellular pollen (Figures 3i-3l), the large cytoplasmic vacuoles disappeared and the cytoplasm showed numerous plastids with starch (Figures 3i, 3k), abundant organelles, endoplasmic reticulum and high ribosome population (Figure 3l). The generative cell was observed detached from the intine and localized in the centre of the pollen grain (Figures 3j, 3l). Mature pollen grains showed very dense cytoplasm and high starch accumulation (Figures 3m-3p); the generative cell and its nucleus exhibited an elongated shape with a highly condensed chromatin (Figure 3n, 3p). At this stage of the gametophyte development, no significant differences were observed in the structure and thickness of the cell wall, except for the formation of specific structures below the apertures (data not shown).

Morphological changes and characterization of sequential developmental stages during microspore-derived embryogenesis

Semithin sections through anthers at the onset of the culture revealed microspores with a large cytoplasmic vacuole and the nucleus pushed towards the exine: the vacuolated microspore stage (Figure 4a). After the inductive treatment, some of the microspores divided producing two cells equivalent in size and structural features, with a dividing cell wall and a low dense cytoplasm with vacuoles (Figure 4b), no two-celled structures with unequal cells were observed in the culture conditions used. Many other microspores apparently died during the treatment or were stopped in their development, exhibiting one nucleus and vacuolated morphology, with no other structures observed at this stage. At later stages of the *in vitro* culture, the two-celled structures proliferated and gave rise to multicellular structures with small cells, having a dense cytoplasm, confined within the pollen wall, the exine (Figure 4c). Then, the exine broke down (Figure 4d) and rounded embryogenic masses or proembryos were released.

At later developmental stages, proembryos displayed highly vacuolated, polygonally-shaped cells with the nucleus in a central position (Figure 4e). There was not starch accumulation indicated by an absence of I_2KI staining (Figure 4h). Later on, morphological changes were observed in the proembryos, probably related to the start of a differentiation. An incipient organization in two layered zones of morphologically different cell types could be observed in these late proembryos without exine. The innermost area contained small, dense cells with small vacuoles (Figure 4f). In the outer zone, bigger cells with large vacuoles were observed (Figure 4f). There was only some low intense starch accumulation in cells of the outer area (Figure 4i).

As embryogenesis proceeded, bigger and more rounded structures, similar to globular embryos were observed (Figure 4g). The different cell types became more evident and a pattern of well defined inner and outer layers of cells was found. There was a higher deposition of starch in the outer layers of the globular embryos than in the external cells of the proembryos (compare Figures 4j and 4i, respectively). At further developmental stages, embryos lost their rounded shape and elongated in some areas (Figure 4k), giving rise to heart-like embryos. At the globular and subsequent stages, organized layers of small, lined-up, isodiametric cells were observed in the outer area. The most peripheral layer of cells showed a morphology resembling a protodermis (Figure 4l), similar to that of zygotic embryos (Figure 4m).

At torpedo stage (Figure 5) different tissues could be recognized: an epidermis (arrows in Figure 5a), the primordium of cotyledon (boxed area b) and a parenchyma (boxed areas c and d). A different distribution of starch between those tissues was found. The parenchymatic cells showed starch accumulation (Figures 5b, 5c and 5d), whereas there was no I_2KI signal in

the epidermis (Figures 5b and 5c) and the cotyledonal primordium (Figure 5b). The presence of starch in the root cap cells (Figure 5d) was also revealed by the cytochemistry assay.

Ultrastructural features of early microspore embryogenesis

In order to identify specific ultrastructural features of the microspore embryogenesis pathway when compared with the gametophytic programme, electron microscopy analysis was performed at defined developmental stages: two-cell structures, multicellular structures with exine, proembryos and globular embryos. The vacuolated microspore at the beginning of the culture showed the typical ultrastructure of the gametophytic pathway (Figure 6a). In response to the inductive treatment, it divided into two similar nuclei (Figure 6b) which were both at a peripheral location close to the exine and next to each other. When compared with the gametophytic pathway, after the mitosis of the microspore, the resulting generative cell was small, attached to the exine and the vegetative cell was bigger with the nucleus more centered and localized in between the generative cell wall and the vacuole (Figure 3e). In comparison with the cell wall in the young bicellular pollen, this suggested that the division plane changed in this sporophytic division. Both embryogenic nuclei showed a similar nuclear organization (Figure 6b). This was opposed to the normal gametophytic development where the vegetative and generative nuclei showed a different pattern (Figures 3f, 3h) that reflects their different activities, cell fate and cell cycle progression. The nuclei of the two-celled structures showed a similar pattern of chromatin condensation (Figure 6b), with small patches of condensed chromatin at the periphery of the cell nucleus, similar to that of the microspore. The large cytoplasmic vacuole, that filled most of the microspore volume, began to be reabsorbed after the induced microspore division with some smaller vacuoles still visible (Figure 6b). Once the microspore was induced towards embryogenesis and the first division

occurs, producing the two equivalent cells, cell proliferation proceeded and gave rise to multicellular structures. The multicellular structures, still confined within the pollen wall (Figure 6c) showed newly-formed and more developed cell walls of different thicknesses, reflecting sequential rounds of division. All cells showed similar ultrastructural organization, with chromatin-decondensed nuclei, active nucleoli and a dense cytoplasm, rich in organelles and vacuoles of different sizes. A differentiated, thick cell wall was observed under the exine at this embryogenic stage, as compared to normally matured pollen grains.

In late proembryos without exine (Figure 7a), two different cell types could be distinguished (Figures 7a-7c). The cells from the inner core were small, with thin, newly-formed cell walls, numerous small vacuoles, nuclei with patches of condensed chromatin and medium-active nucleoli, often more than one per cell (Figure 7b). In the outer layers, the cells were bigger, with thick and differentiated cell walls, large vacuoles, plastids with starch, a decondensed pattern of chromatin and highly active nucleoli (Figure 7c). In globular embryos (Figure 7d), similar differences between cell types were also observed together with some specific features of this stage. The inner cells showed dense cytoplasm, very few and smaller vacuoles and large nucleus (Figure 7e), resembling the organization of a proliferating active cell. A specific feature found at this stage was that the cells from the peripheral layers contained numerous and large amyloplasts, with several starch grains inside the plastids, big vacuoles, and small nucleus (Figure 7f).

DISCUSSION

Heat-treated *in vitro* anther cultures regenerate haploid plants and constitute a suitable system for cellular studies on the embryogenesis process

The earliest studies on the production of haploid plants in *Capsicum* through *in vitro* anther cultures date back to 1979 (Sibi *et al.* 1979). Embryogenesis was triggered by pre-treatment of the anthers at 4ºC for 48 hours, then cultured in a base medium with hormones and vitamins. In this work, haploid plants of *Capsicum annuun* have been regenerated from *in vitro* anther cultures in a new variety, Yolo Wonder B. A heat treatment at 35ºC for 8 days has been demonstrated to be well suited as inductive treatment, like for other pepper genotypes, for the anther response within the range of other low-responsive cultivars of pepper (Mitykó *et al.* 1995). This inductive treatment in pepper is longer than the heat shock used to induce microspore embryogenesis in other species (Custers *et al.* 1994). Rooting and acclimatization was achieved, although the haploid plants grew less vigorously than the diploid control plants.

The study of the cellular changes related to pollen embryogenesis in anther cultures of *Capsicum* constitutes a difficult task due to the low yield of induction. The embryos emerging from the anthers were considered to be the signal of a positive response to the inductive treatment, about 20 to 30 days after setting the culture. The asynchrony of embryogenesis in anther cultures (Bueno *et al.* 2000, Ramírez *et al.* 2004) represented an advantage for cellular studies since it permitted the identification of sequential developmental stages on consecutive sections across single anthers, making the characterization of the earliest events, along with further stages of development possible. The study of cellular markers of pollen

embryogenesis and subsequent development was approached by fixing and low-temperature processing of anthers with emerging embryos, at different time points of the culture, which resulted in a good ultrastructural preservation of the anthers and the embryogenic structures.

Defined changes in the cell organization determine cellular markers of the switch to the sporophytic pathway

The determination of early differential markers between the gametophytic and the embryonic pathways can be used to define the processes that are activated and/or repressed when the microspores change their developmental programme in response to a stress treatment (Testillano *et al.* 2000, Ramírez *et al.* 2004, Seguí *et al.* 2003, 2005). The occurrence of defined cell changes at specific developmental stages was related to the activation of proliferation and the beginning of differentiation (Coronado *et al.* 2002, Ramírez *et al.* 2004, Seguí *et al.* 2005). The analysis reported here showed defined rearrangements in specific subcellular compartments, i.e. amyloplasts, vacuoles, cell walls and nuclei.

Starch distribution

There was no starch accumulation in young proembryos during the early events of microspore embryogenesis in *C. annuum*. No starch deposition was found, not only after induction under starvation, as in tobacco (Vicente *et al.* 1991), but also by a heat treatment, as in rapeseed (Testillano *et al.* 2001, Seguí *et al.* 2003), and pepper (this work). Therefore, the lack of starch is not a consequence of sucrose fasting. Early starch accumulation in microspore proembryos still surrounded by the exine has only been found in a few reports on cereals (Indrianto *et al.* 2001, Testillano *et al,* 2002) and it has been suggested as a specific feature of monocot species. Since starch deposition could be associated to differentiation, the

early events of microspore-derived embryogenesis could be related to a previous stage before the entrance to differentiation.

Deposits in vacuoles

Electron dense deposits in the vacuoles have been reported as markers of pollen embryogenesis in some species (Sangwan and Camefort 1983). Nevertheless, no evidence supporting this hypothesis has been found in *Capsicum annuum*. In these species, after fixation with glutaraldehyde, dense deposits were not found either in young proembryos or during pollen formation and maturation (Fadón 1993; González-Melendi *et al.* 1995).

Symmetric division of induced cells

It is widely accepted that the first morphological evidence of the embryogenic pathway is the symmetric division of the microspore, as opposed to the asymmetric division occurring during the normal gametophytic development (Zaki and Dickinson 1991, Hause and Hause 1996). In pepper, different division patterns at the onset of microspore embryogenesis have been reported, including asymmetric divisions (Kim *et al.* 2004). These embryogenic pathways were observed after different inductive treatments and culture conditions and were the result of the response of either the vegetative or the generative nuclei of the bicellular pollen (Kim *et al.* 2004). Different division patterns were also reported on isolated microspore cultures of pepper (González-Melendi *et al.* 1995, 1996). On the other hand, the late vacuolated microspore was the most responsive stage, not only for pepper (González-Melendi *et al.* 1995) but for many other species (Bueno *et al.* 2000, Testillano *et al.* 2002, Pechan *et al.* 1991). The cellular and ultrastructural analysis performed here showed that in anther cultures beginning with the vacuolated microspore, an equal division took place. Different division

patterns have been described but their successful embryogenic progress has not yet been inequivocally proven.

Cytokinesis and cell wall differentiation

Multicellular proembryos, still surrounded by the exine, showed completely walled-off cells with cell walls of different thicknesses reflecting different division rounds. The development of a thick cell wall underneath the exine in multicellular proembryos constitutes a specific and differential feature of this early developmental stage, which was also found in other systems (González-Melendi *et al.* 1995, 1996, Ramírez *et al.* 2001, 2004). Further investigations are needed to determine the components and distribution of this thick wall. During developmental processes, the structure and components of the cell walls change (Catoire *et al.* 1998); many molecular markers of somatic embryogenesis and organogenesis have been found in cell walls (Fortes *et al.* 2002, Fry *et al.* 1993). The development of this thick wall at this particular stage of microspore embryogenesis could be in relation to the initiation of the subsequent differentiation events, which only occur after the release from this wall.

Nuclear subcompartments

The nucleus is highly dynamic and the architecture of its functional domains (condensed chromatin, interchromatin region and nucleolus) changes in response to nuclear activity (Raska 1995, Dundr and Mistelli 2001, Risueño and Medina 1986). In the bicellular pollen, the generative and vegetative nuclei are different, dimorphism which infers a differential cell activity, fate and cell cycle progression between both cells (González-Melendi *et al.* 2000, McCormick 1993). The ultrastructural study performed here showed that in pepper, as in other species, the active metabolism of the vegetative cell is mirrored by its nucleus, with

dispersed chromatin and an active nucleolus, whereas the generative nucleus shows features of low transcriptional activity such as condensed chromatin patches and a compact nucleolus. In contrast, the two nuclei resulting from the symmetric division showed a similar pattern of chromatin, with small patches of condensed chromatin at the periphery of the nucleus (Testillano *et al.* 2000, 2005). The architecture of the nucleolus can also be used as a marker of its activity (Risueño *et al.* 1988). Immediately after the symmetric division the nucleoli are compacted, indicating the resuming of the transcriptional activity occurring in G1 phase (Risueño *et al.* 1982; Risueño and Medina 1986). Remodeling of the functional organization of nuclear domains has been reported as a main event occurring in the switch of differentiating plant cells towards proliferation (Testillano *et al.* 2005).

Young multicellular proembryos showed nuclei with dispersed chromatin pattern and active nucleoli and a dense cytoplasm with small vacuoles. Similar ultrastructural features have been reported for cells in proliferation (Risueño and Moreno Díaz de la Espina 1979; Risueño *et al.* 1982). Differential expression of MAP kinases with roles in proliferation and differentiation has been observed in consecutive developmental stages during microspore embryogenesis in rapeseed (Seguí *et al.* 2005) and tobacco (Coronado *et al.* 2002). Once the proembryos have been released from the exine, the observation of two different cell types between the inner core and the outer layers indicates the starting of a cell differentiation.

Further microspore-derived embryo development mimics zygotic embryogenesis

The structural pattern of development of microspore-derived embryos is poorly understood. Apart from rapessed, its comparison with the formation of the zygotic embryo has been very scarcely described (Yeung *et al.* 1996). The results presented in pepper showed that the initial proliferation and the formation of multicellular structures and proembryos do

not seem to follow a regular and defined pattern of divisions, as the zygotic embryogenesis (Raghavan 2000). At further developmental stages, the differentiation of a protodermis in globular embryos and the ulterior development of heart-like and torpedo embryos mimic the main events of zygotic embryo formation. Also, the organization of the root meristems and the presence of starch in the root cap cells are similar in zygotic and microspore embryos. Further work, now in progress, will help to define more precisely the pattern of differentiation followed by microspore-derived embryos and its comparison with the zygotic pathway. This suggests that the proliferative structures resulting from induction adquire the embryogenic competence.

In summary, the cellular characterization of pollen embryogenesis in *Capsicum annum* L. showed defined subcellular rearrangements affecting the nucleus, starch storage, vacuoles and the cell wall at specific developmental stages. They accompanied proliferation and differentiation events and could be considered as possible markers for early monitoring of microspore embryogenesis. This knowledge, in a horticultural crop of agronomic interest, like pepper, could guide future strategies for improving the yield of the process and its use in transformation and breeding through double-haploid plants.

MATERIALS AND METHODS

Materials

Seeds of *Capsicum annuum* L., var Yolo Wonder B (Ramiro Arnedo, S. A., Calahorra, La Rioja, Spain) were germinated in soil and the plants grown in a growth chamber (MLR 350H, SANYO) at 25ºC, 80% humidity with a photoperiod of 16 hours of light. Buds of different sizes were selected and anthers were excised and used for either microscopical analysis of normal gametophytic development or *in vitro* culture. Some ovaries were also excised from open flowers for microscopical study of zygotic embryos.

In vitro anther culture

Only buds from the first flowering were used for the cultures. Pollen development stage was determined by DAPI staining on anther squashes (Vergne *et al.* 1987, González-Melendi *et al.* 1995). Anthers with blue-colored tips from buds with a similar length of petals and sepals, corresponding to the late vacuolated microspore were selected for the cultures. The buds were surface sterilized with 5 % Calcium hypochlorite. Fifteen anthers were plated on each 5 cm diameter Petri dish containing Cp induction medium (Dumas de Vaulx *et al.* 1981) supplemented with 0.01 mg/l 2,4–dichlorophenoxyacetic acid (2,4-D) and kinetin. After 8 days at 35ºC in darkness, the cultures were transferred to 25ºC with a photoperiod of 12 hours of light at 14,000 luxes (186 μmol/m^2/s). After 12 days of induction in Cp medium, anthers were transferred to R1 medium (Dumas de Vaulx *et al.* 1981) supplemented with 0.1 mg/l of kinetin. Plantlets were grown in a V3 hormone-free medium for further development (Dumas de Vaulx *et al.* 1981).

Flow cytometry

To determine the ploidy level of the regenerated plantlets we used flow cytometry (Dolezel *et al.* 1989). Nuclei were isolated by lacerating leaves in a lysis buffer (15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl and 0.1% Triton X-100, pH 7.5), which was filtered through a 30 µm nylon mesh. The filtrate was transferred to 2 ml eppendorf tubes and centrifuged at 600 g for 20 min at 4ºC, the pellet was resuspended in 0.5 ml of lysis buffer. The nuclei suspension was transferred to cytometer tubes with 55 µl of solution of Propidium Iodide (1 mg/ml) in PBS containing 15 mg/ml of RNase. 10000 nuclei of each sample were measured in an Epics-xl flow cytometer (Coulter). As a control we used a nuclei suspension from diploid parental plants.

Sample cryoprocessing and electron microscopy

Anthers at different developmental stages throughout gametogenesis, at different time-points of the anther culture and macroscopic embryos emerging from the anthers were fixed overnight at 4ºC in 4% formaldehyde in phosphate buffered saline (PBS), pH 7.3. Then, they were washed in PBS and dehydrated through a methanol series (30%, 50%, 70% and 100%) with a progressive lowering of temperature, from 4ºC to –30ºC. The specimens were infiltrated in mixtures methanol:Lowicryl K4M (in a series 2:1, 1:1 and 1:2) at –30ºC, embedded in pure resin at the same temperature and polymerized under U.V. light at -30ºC in a Leica AFS device, as previously described (Testillano *et al.* 1995). 1 µm (semithin) and 100 nm (ultrathin) sections were obtained and used for light and electron microscopy observations. Toluidine-blue stained semithin sections were observed under bright field for structural analysis. Ultrathin sections were counterstained with 5% uranyl acetate and 1 % lead citrate and observed in a JEOL 1010 microscope at 80 KV for general ultrastructure description.

Cytochemical staining

Starch was detected by I_2KI staining (O'Brien and McCully 1981) on semithin sections and observed under bright field. DAPI staining for DNA was applied to semithin sections (Testillano *et al.* 1995) and observed under UV light in an epifluorescence microscope.

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REFERENCES

Bárány, I., Testillano, P.S., Mitykó, J., and Risueño, M.C. (2001) The switch of the microspore developmental programme in *Capsicum* involves HSP70 expression and leads to the production of haploid plants. Int. J. Dev. Biol. **45,** S39-S40.

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

Boluarte-Medina T and Veilleux R.E (2002) Phenotypic characterization and bulk segregant analysis of anther culture response in two backcross families of diploid potato. Plant Cell Tissue Organ Cult. **68,** 277-286.

Bueno, M.A., Gómez, A., Boscaiu, M., Manzanera, J.A., and Vicente,O. (1997) Stress induced haploid plant production from anther cultures of *Quercus suber*. Physiol. Plant. **99,** 335-341.

Catoire, L., Pierron, M., Morvan, C., du Penhoat, C.H., and Goldberg, R. (1998) Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR spectroscopy. Implications In cell wall expansion. J. Biol. Chem. **273,** 33150-33156.

Chani, E., Veilleux, R.E., and Boluarte-Medina, T. (2000) Improved androgenesis of interspecific potato and efficiency of SSR markers to identify homozygous regenerants. Plant Cell Tissue Organ Cult. **60,** 101-112.

Chupeau, Y., Caboche, M., and Henry, Y. (1998). Androgenesis and haploid plants., Springer-Verlag, Berlin, Heidelberg.

Coronado, M.J., González-Melendi, P., Seguí, J.M., Ramírez, C., Bárány, I., Testillano, P., and Risueño, M.C. (2002) MAPKs entry into the nucleus at specific interchromatin domains in plant differentiation and proliferation processes. J. Struct. Biol. **140,** 200-213.

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

Dolcet-Sanjuan R, Claveria E, and Huerta A (1997) Androgenesis in *Capsicum annuum* L. Effects of carbohydrate and carbon dioxide enrichment. J. American Society for Horticult. Sci. **122,** 468-475.

Dolezel, J., Binarova, P., and Lucretti, S. (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol. Plant. **31,** 113-120.

Dumas de Vaulx, R., Chambonnet, D., and Pochard, E. (1981) Culture *in vitro* d'anthères de piment (*Capsicum annuum* L.): amélioration des taux d'obtention de plantes chez différents génotypes par des traitements à +35ºC. Agronomie **1,** 859-864.

Dundr, M. and Misteli, T. (2001) Functional architecture in the cell nucleus. Biochem. J. **356,** 297-310.

Fadón, B. (1993) Estudio ultraestructural y detección *in situ* de macromoléculas durante el desarrollo del polen de *Capsicum annuum* L. en relación con el proceso de inducción de la androgénesis. Doctoral Thesis. Univ. Compl. Madrid 1-377.

Fortes, A.M., Testillano, P.S., Del Carmen, R.M., and Pais, M.S. (2002) Studies on callose and cutin during the expression of competence and determination for organogenic nodule formation from internodes of *Humulus lupulus* var. Nugget. Physiol. Plant. **116,** 113-120.

Franchi, G.G., Bellani L, Nepi M, and and Pacini E. (1996) Types of carbohydrate reserves in pollen: localization, systematic distribution and ecophysiological significance. Flora **191,** 143-159.

Fry S, Aldington S, Hetherington P, and Aitken J (1993) Oligosaccharides signals and substrates in the plant cell wall. Plant Physiol. **103,** 1-5.

Germanà, M.A., Crescimanno, F.G., De Pasquale, F. and Wang, Y.Y. (1991). Androgenesis In 5 cultivars *of Citrus limon* L. *Burm.f.* Acta Hort. **300,** 315-324

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

González-Melendi, P., Testillano, P.S., Ahmadian, P., Fadón, B., Vicente, O., and Risueño, M.C. (1995) *In situ* characterization of the late vacuolate microspore as a convenient stage to induce embryogenesis in *Capsicum*. Protoplasma **187,** 60-71.

González-Melendi, P., Testillano, P.S., Ahmadian, P., Reyes, J., and Risueño, M.C. (2000) Immunoelectron microscopy of PCNA as an efficient marker for studying replication times and sites during pollen development. Chromosoma **109,** 397-409.

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

Guha-Mukherjee, S. (1999) The discovery of haploid production by anther culture. *In vitro* Cell Dev. Biol. -Plant **35,** 357-360.

Gyulai, G., Gémesné, J.A., Sági, Z., Venczel,G., Pintér,P., Kristóf, Z., Törjék, O., Heszky,L., Bottka, S., Kiss, J., and Zatykó, L. (2000) Doubled haploid development and PCR-analysis of F-1 hybrid derived DH-R-2 paprika (*Capsicum annuum* L.) lines. J. Plant Physiol. **156,** 168-174.

Hause, G. and Hause, B. 1996. Induction of embryogenesis in isolated microspores and pollen of *Brassica napus* L. Wageningen Agricultural University, Wageningen, The Netherlands.

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

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

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

Kumlehn, J. and Lorz, H. (1999) Monitoring sporophytic development of individual microspores of barley (*Hordeum vulgare* L.). In Anther and Pollen: from Biology to Biotechnology (Clement,C., Paccini,E., and Audran,J.C., eds.), pp 183-190, Springer Verlag, Berlin, Heilderberg, New York

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

Mitykó, J., Andrásfalvy, A., Csilléry, G., and Fári, M. (1995) Anther culture response in different genotypes and F1 hybrids of pepper (*Capsicum annuum* L.). Plant Breeding **114,** 78-80.

Mitykó, J., Szabó, L., and Barnabás, B. (1999) Colchicine induced ultrastructural changes in barley and pepper microspores. J. Slovak Academy of Sciences **54,** 24-25.

O'Brien, T.P. and McCully, M.E. (1981). 'The study of plant structure. Principles and selected methods. Termarcarphi, Wantirna, Victoria, Australia.

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

Raghavan, V. (1986). Embryogenesis in angiosperms. A developmental and experimental study, Cambridge University Press, Cambridge, London.

Raghavan, V. (2000). Developmental Biology of Flowering plants, Springer-Verlag, New York.

Ramírez, C., Testillano, P.S., Castillo, A.M., Vallés, M.P., Coronado, M.J., Cistué, L., and Risueño, M.C. (2001) The early microspore pathway in barley is accompanied by concrete ultrastructural and expression changes. Int. J. Dev. Biol. **45,** S57-S58.

Ramírez, C., Testillano, P.S., Pintos, B., Moreno-Risueño M.A., Bueno. M.A, and Risueño MC (2004) Changes in pectins and MAPKs related to cell development during early microspore embryogenesis in *Quercus suber* L. Eur. J. Cell. Biol. **83,** 213-225.

Raska,I. (1995) Nuclear ultrastructures associated with the RNA synthesis and processing. J. Cell Biochem. **59,** 11-26.

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

Risueño, M.C. and Medina, F.J. (1986). The nucleolar structure in plant cells. Cell Biol. Rev. **7**, 1-140.

Risueño, M.C., Medina, F.J., and Moreno, S. (1982) Nucleolar fibrillar centers in plant meristematic cells: ultrastructure, cytochemistry and autoradiography. J. Cell Sci. **58,** 313-329.

Risueño, M.C. and Moreno Díaz de la Espina (1979) Ultrastructural and cytochemical study of the nucleus of the dormant root meristematic cells. J. Submicros. Cytol. **11,** 85-95.

Risueño, M.C., Testillano, P.S., and Sánchez-Pina, M.A. (1988) Variations of nucleolar ultrastructure in relation to transcriptional activity during G1, S, G2 of microspore interphase. In Sexual Reproduction in Higher Plants (Cresti,M., Gori,P., and Paccini,E., eds.), pp 9-14, Springer-Verlag, Berlin. Heidelberg

Rokka, V.M., Pietila, L., and Pehu, E. (1996) Enhanced production of dihaploid lines via anther culture of tetraploid potato (*Solanum tuberosum* L ssp *tuberosum*) clones. Amer. Potato J. **73,** 1-12.

Sangwan, R.S. and Camefort, H. (1983) The tonoplast, a specific marker of embryogenic microspores of *Datura* cultured *in vitro*. Histochemistry **78,** 473-480.

Seguí J.M., Testillano, P.S., Jouannic, S., Henry, Y., and and Risueño M.C (2005) MAP kinases are developmentally regulated during stress-induced microspore embryogenesis in *Brassica napus* L. Histochem. Cell Biol. In the press.

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

Shtereva, L.A., Zagorska, N.A., and Dimitrov, B.D. (1998) Induced androgenesis in tomato (*Lycopersicum esculentum* Mill). II. Factors affecting induction of androgenesis. Plant Cell Rep. **18,** 312-317.

Sibi, M., Dumas de Vaulx, R., and Chambonnet, D. (1979) Obtention de plantes haploïdes par androgenèse *in vitro* chez le piment (*Capsicum annuum* L.). Ann. Amélior. Plantes **29,** 583-606.

Teparkum, S. and Veilleux, R.E. (1998) Indifference of potato anther culture to colchicine and genetic similarity among anther-derived monoploid regenerants determined by RAPD analysis. Plant Cell Tissue Organ Cult. **53,** 49-58.

Testillano, P., Ramírez, C., Domenech, J., Coronado, M.J., Vergne, P., Matthys-Rochon, E., and Risueño, M.C. (2002) Young microspore-derived maize embryos show two domains with defined features also present in zygotic embryogenesis. Int. J. Dev. Biol. **46,** 1035-2047.

Testillano, P.S., Coronado, M.J., Bárány, I., Latino, A., Seguí, J.M., Ramírez, C., Díaz-Mendoza, M., and Risueño, M.C. (2001) Defined cellular markers including specific MAPK distribution in two pollen developmental programmes. In Microscopy, Barcelona 2001, pp 68-69, Universitat de Barcelona, Barcelona

Testillano, P.S., Coronado, M.J., Seguí, J.M., Domenech, J., González-Melendi, P., Raska,I., and Risueño, M.C. (2000) Defined Nuclear Changes Accompany the Reprogramming of the Microspore to Embryogenesis. J. Struct. Biol. **129,** 223-232.

Testillano, P.S., González-Melendi, P., Ahmadian, P., Fadón, B., and Risueño, M.C. (1995) The immunolocalization of nuclear antigens during the pollen developmental program and the induction of pollen embryogenesis. Exp. Cell Res. **221,** 41-54.

Testillano, P.S., González-Melendi, P., Coronado, M.J., Seguí, J.M., Moreno, M.A., and Risueño, M.C. (2005) Differentiating plant cells switched to proliferation remodel the functional organization of nuclear domains. Cytogenet. Genome Res. **109**. 10.1159/000082396.

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

Vergne, P., Delvallee, I., and and Dumas C (1987) Rapid assessment of microspore and pollen development stage in wheat and maize using DAPI and membrane permeabilization. Stain Tech. **72,** 299-304.

Vicente, O., Benito-Moreno, R.M., and Heberle-Bors,E. (1991) Pollen cultures as a tool to study plant development. Cell Biol. Rev. **25,** 295-305.

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

Zagorska, N.A., Shtereva, L.A., and Dimitrov, B.D.K.M.M. (1998) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.) I. Influence of genotype on androgenetic ability. Plant Cell Rep. **17,** 968-973.

Zagorska, N.A., Shtereva, L.A., Kruleva, M.M., Sotirova, V.G., Baralieva, D.L., and Dimitrov, B.D. (2004) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.). III. Characterization of the regenerants. Plant Cell Rep. **22,** 449-456.

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

Zamir D, Jones R.A, and Kedar N (1980) Anther culture of male sterile tomato (*Lycopersicon esculentum* Mill.) mutants. Plant Sci. Lett. **17,** 353-361.

Ziv M, Hedary D, and Kedar N (1984) *Lycopersicon esculentum:* trifoliate plants recovered from anter cultured of heterozygous *tftf* plants. Plant Cell Rep. **3,** 10-13.

FIGURE LEGENDS

Figure 1: Anther culture and haploid plants regeneration. a: Anther at the onset of the culture; b: Anther after 6 days in culture; c, d: Embryos emerging from the anthers after 30 days in culture, showing roots (c) and shoots (d); e, f, g: Plantlets with cotyledons (e) and with leaves (f, g) subcultured in growing medium; (h) 80 days-old regenerated haploid plant from anther culture (left-hand side) and a diploid control of the same age (right-hand side). Bars in a - d: 2. 5 mm; in e - h: 5 mm.

Figure 2: Flow cytometry analysis. Charts showing the relative amounts of DNA after propidium iodide staining of a population of leaf nuclei. a: Haploid regenerated plantlets from *in vitro* anther culture (G1 peak was set at channel 100). b: Double-haploid regenerated plantlet from *in vitro* culture (G1 peak was set at channel 200). c: diploid control. In (d) haploid and diploid samples are prepared and measured together, showing two G1 peaks, at 1C and 2C respectively and a smaller one (G2) at 4C. The *x*-axis represents the log of absorbance of propidium iodide (PI) and the *y*-axis represents the number of nuclei.

Figure 3: Normal gametophytic development as a control of the embryogenic pathway. a-d: vacuolated microspore, e-h: young bicellular pollen, i-l: mid bicellular pollen, m-p: mature pollen. Semithin sections observed under phase contrast (a, e, i, m), fluorescent microscopy after DAPI staining for DNA (b, f, j, n, arrows: generative nuclei) and bright field after I_2KI staining for starch (c, g, k, o). d, h, l, p: electron micrographs showing the main ultrastructural features of the different developmental stages. Ex: exine, V: vacuole, N: nucleus, Nu: nucleolus, Chr: condensed chromatin, Ct: cytoplasm, GN: generative nucleus,

VN: vegetative nucleus, S: starch. Bars in light micrographs: 10 μm, and in electron micrograph: 1 μm.

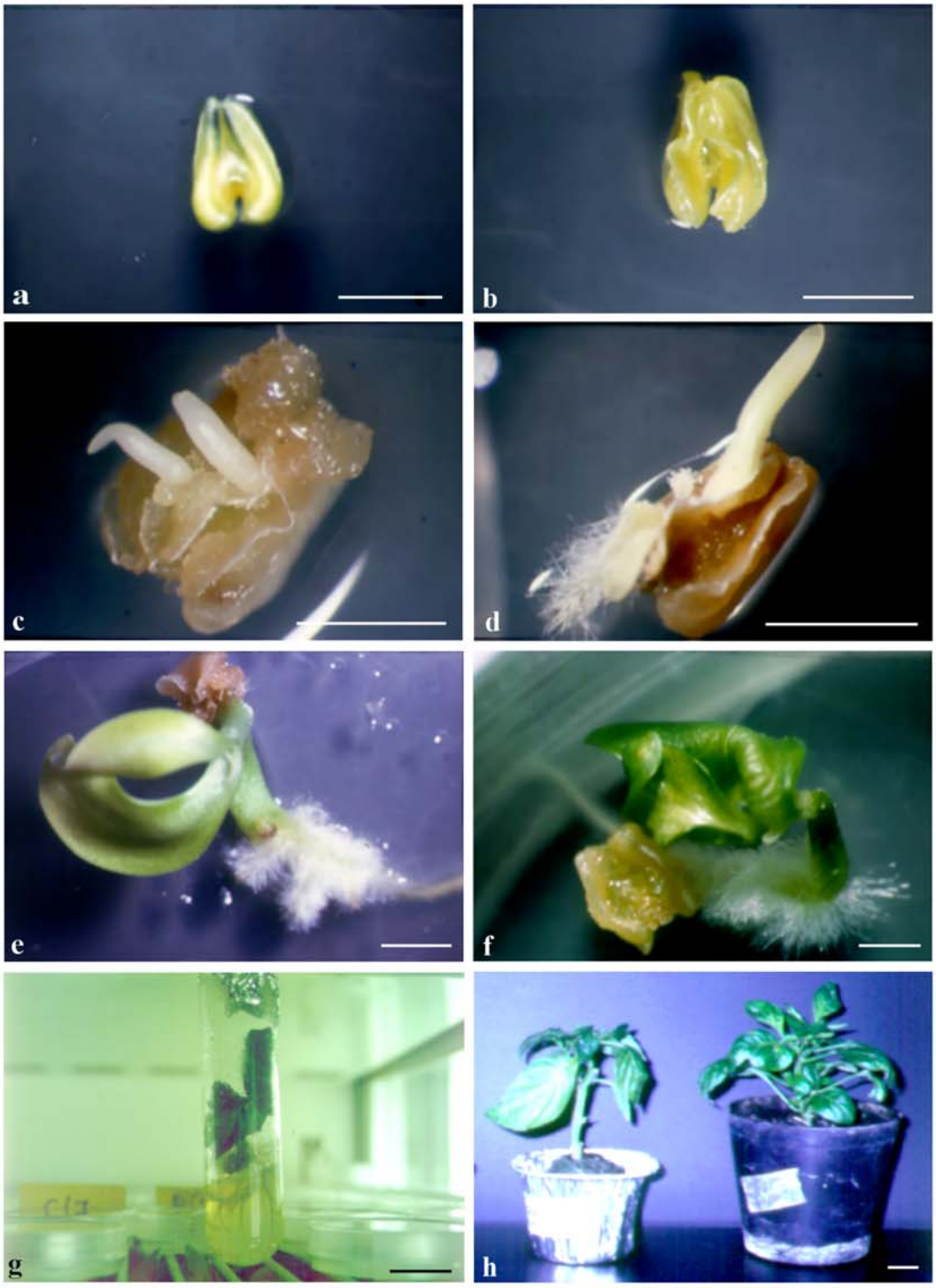
Figure 4: First stages of microspore-derived embryogenesis. Cellular organization (a-g, k-l) and starch cytochemistry (h-j). Semithin sections observed in a light microscope. a: Vacuolated microspores contained in the anthers at the time of setting the cultures; b: Two-celled proembryos, showing similar cells and the formation of a cell wall between them; c: Multicellular proembryos surrounded by the exine (Ex); d: Multicellular proembryos being released as the exine (Ex) breaks down (arrows); e - j: Progression from multicellular proembryos to globular embryos, observed under phase contrast (e - g) and bright field after I_2KI staining to reveal starch (h – j). k: Heart-like microspore-derived embryo showing a peripheral protodermis. l-m: High magnification micrographs showing the protodermis of microspore-derived (l) and zygotic (m) embryos. Bars: 10 μm.

Figure 5: Microspore-derived embryo at torpedo stage. Semithin sections observed at the light microscope, under phase contrast (a) and bright field after I_2KI staining to reveal starch (b – d). a: Different tissues can be recognized at this stage: the epidermis (arrow), the primordium of cotyledon (boxed area b) and the parenchyma (boxed areas c and d). I_2KI staining showed starch accumulation mainly in parenchymatic cells (b and c) but not in the cotyledon primordium (central area in b). Also, an accumulation of starch in the cap cells of the root meristems can be observed (d). Bars: 10 μm.

Figure 6: Ultrastructure of early microspore embryogenesis. **a)** Vacuolated microspore with the nucleus (N) at the periphery and a large vacuole (V). **b)** Two-celled proembryo, resulting from a symmetric division of the microspore, with two nuclei (N) showing a similar

organization pattern. **c)** Multicellular proembryo within the exine and a thick cell wall (*) developing underneath it. Nu: nucleolus, Chr: condensed chromatin, arrow: nuclear body, Ct: cytoplasm,V: vacuole, Cw: cell wall, Ex: exine. Bars: 1 μm.

Figure 7: Ultrastructure of microspore-derived proembryos (a-c) and globular embryos (d-f). a, d: General structure of the proembryos (a) and globular embyos (d) under the light microscope. b, c, e, f: Ultrastructural features revealed by the electron microscope of the inner (b) and outer (c) cells of the proembryo, and the inner (e) and outer (f) cells of the globular embryo. Large vacuoles (V) and starch (S) granules in large amyloplasts are observed in the outer cells of globular embryos. Ct: cytoplasm, N: nucleus, Nu: nucleolus, Cw: cell wall. Bars in a, d: 20 μm, in b, c, e, f: 1 μm.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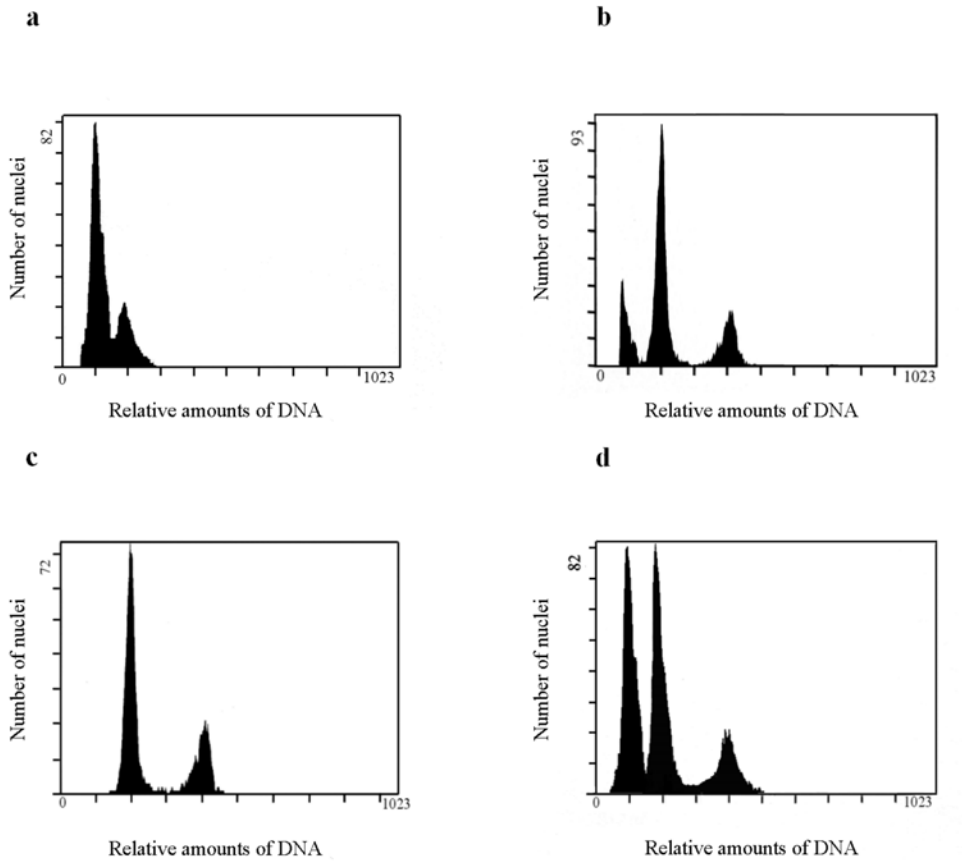
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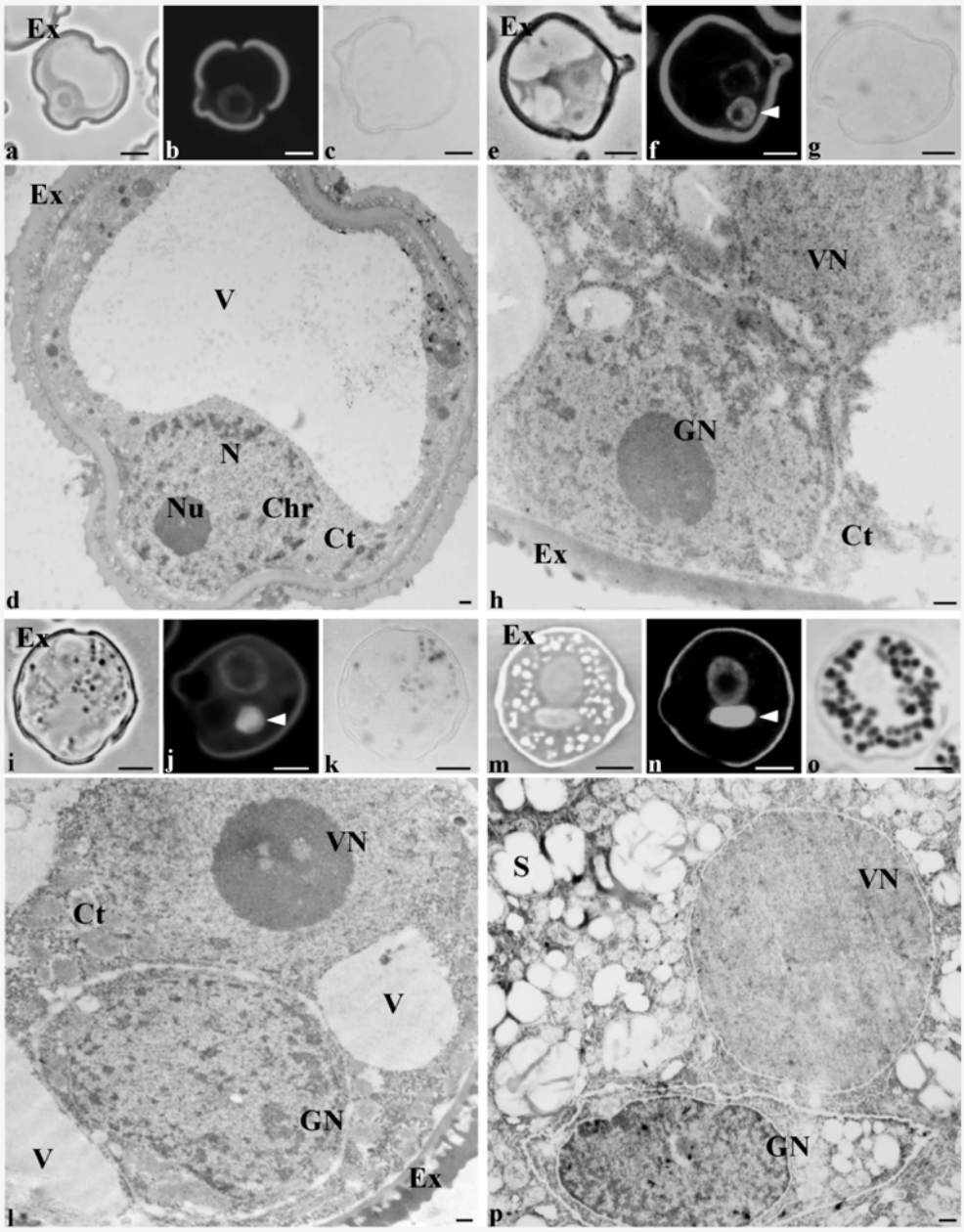
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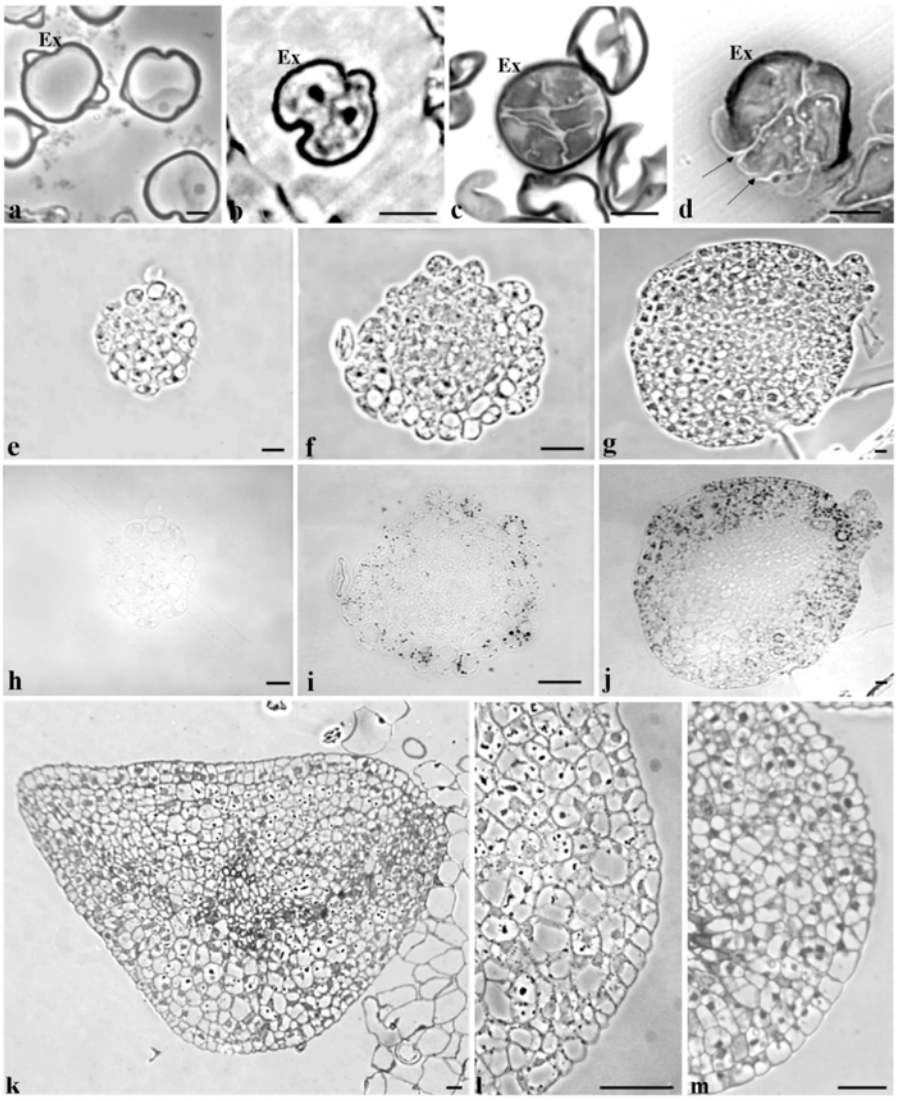
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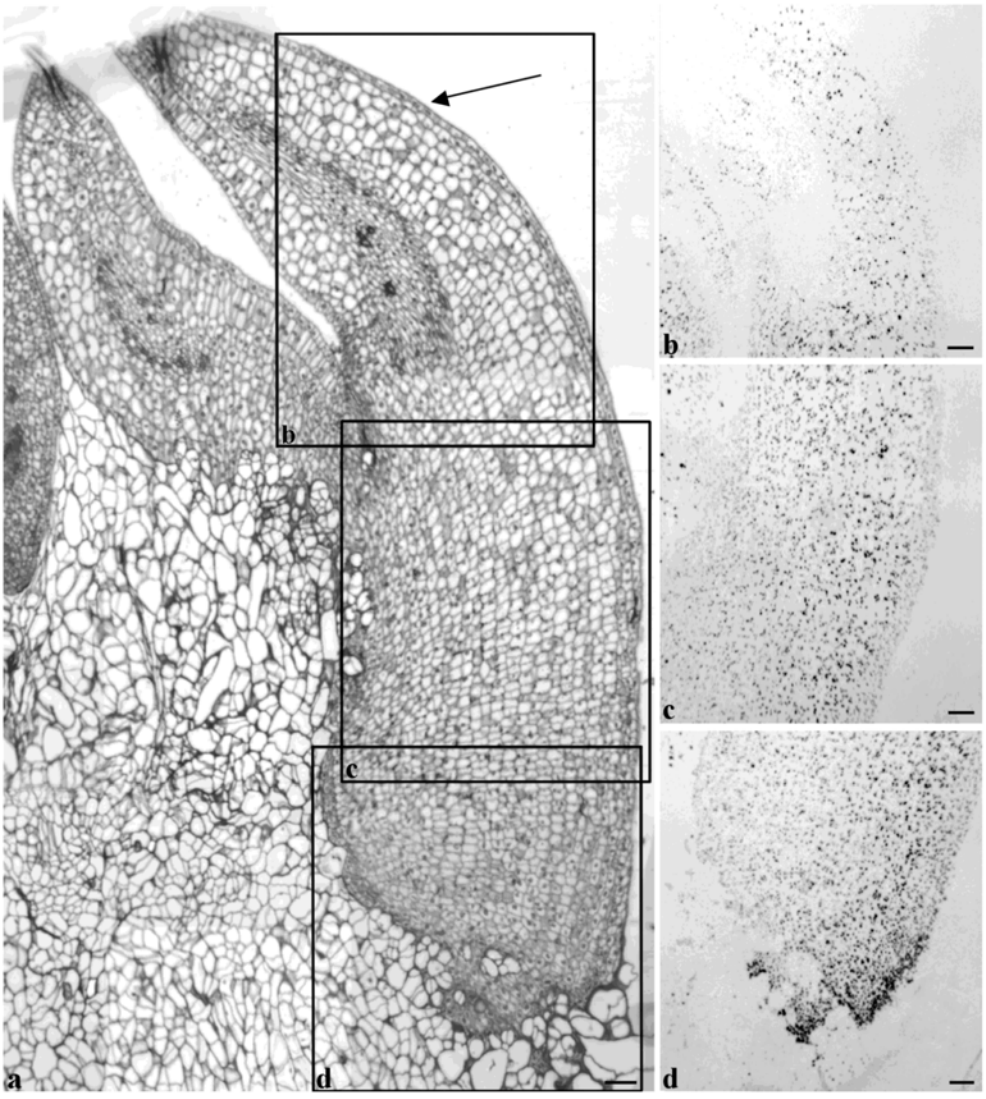
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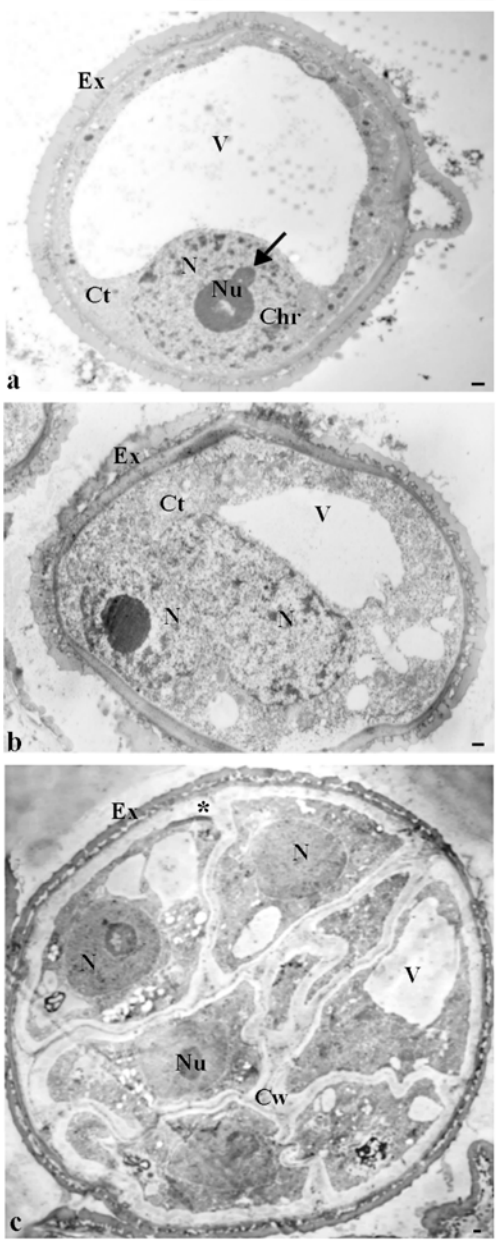
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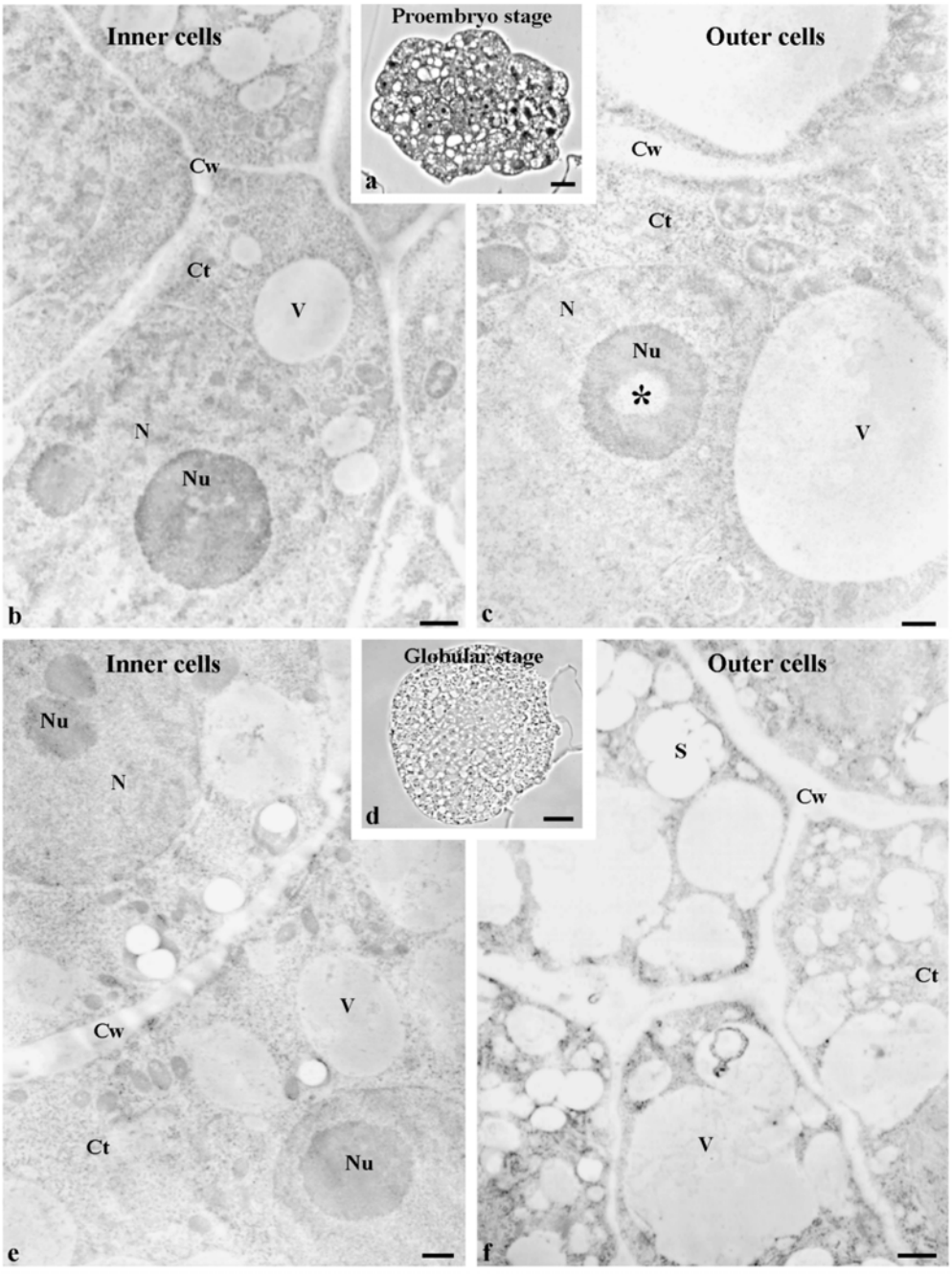
Proembryo stage
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