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CELL ARCHITECTURE DURING GAMETOPHYTIC AND EMBRYOGENIC MICROSPORE DEVELOPMENT IN *BRASSICA NAPUS* L.

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ABSTRACT

Under controlled conditions *in vitro*, the microspore can follow different developmental programmes. After a stress treatment, responsive microspores deviate from its gametophytic pathway towards embryogenesis to form haploid embryos and plants. Other microspores, not sensitive to induction, follow a gametophytic-like pathway *in vitro*. In *Brassica napus* L., a model for pollen embryogenesis, the inductive treatment consists on 32ºC for at least 8 hours. If microspore culture is kept at 18ºC, gametophytic development is mimicked *in vitro*. The microspore reprogramming to embryogenesis involves defined changes affecting cell activities and structural organization which can be considered as markers of the microspore embryogenic pathway. Less is known about others developmental programmes followed by the microspore *in vitro* after both, inductive and non-inductive conditions. In this work, the cell architecture of the microspore following different developmental pathways *in vitro* have been compared with the gametophytic development *in vivo* in *Brassica napus* , at both light and electron microscopy level. Low-temperature processing of the samples, cytochemical and immunocytochemical approaches to identify various cell components have been performed. Differences in specific cellular features such as cellular size and shape, nuclear architecture, starch accumulation, presence of vacuoles and ribosomal population were studied to characterize sequential stages of microspore embryogenesis and other pathways occurring *in vitro*. The presence of

abundant starch grains in a defined cytoplasmic region appeared as a specific feature of the *in vitro* non-embryogenic development of the microspore, occurring under both, inductive and non-inductive conditions.

INTRODUCTION

The immature pollen grain, the microspore, under stress conditions *in vitro*, can switch its developmental programme towards embryogenesis by proliferation and to form haploid embryos and plants (Chupeau *et al.* 1998). In most species, the reprogramming of the microspore is only possible at specific developmental stages such as the late vacuolate microspore (González-Melendi *et al.* 1995, 1996). The comparison between the gametophytic and sporophytic pathways followed by the microspore, permitted to analyse the subcellular changes in plant differentiating cells when switch to proliferation. The search of molecular and cellular markers during early stages of microspore embryogenesis constitutes an important goal to monitor the physiological processes involved in the induction and to identify cells committed to the new developmental programme. This reprogramming is accompanied by defined changes affecting various cell activities and structural organization of subcellular compartments which can be considered as markers of the pollen reprogramming process. (Seguí-Simarro 2001, Barany *et al.* 2005, Testillano *et al.* 2005).

In *Brassica napus* L., a dicot species which can be considered as a model for pollen embryogenesis induction, the stress inductive treatment consists on 32ºC for at least 8 hours (Custer *et al.* 1994). After induction *in vitro*, some microspores switch to embryogenesis whereas others, not sensitive to induction follow other pathways, still largely unknown. If microspore culture is kept at 18ºC, gametophytic development is followed *in vitro* (Custers *et al.* 1994). Several studies have analyzed different aspects of the stress-induced microspore embryogenic development *in vitro* (Cordewener *et al.* 1995, Seguí-Simarro 2001, Segui-Simarro *et al.* 2003, 2005, Yeung *et al.* 1995, Hause and Hause 1996, Straatman and Schel 1997), but much less attention has been devoted to others developmental programmes followed by the microspore *in vitro* after both, inductive and non-inductive conditions.

In this work, the cell architecture of the microspore switched to embryogenesis and the different developmental pathways followed *in vitro* have been compared with the gametophytic development *in vivo* and *in vitro* in *Brassica napus* , at both light and electron microscopy level. Low-temperature processing of the samples, to preserve their structural and biochemical integrity, specifically cryoembedding in Lowicryl K4M was

performed, followed by cytochemical and immunocytochemical approaches to identify specific cell components. Differences in specific cellular features such as cellular size and shape, nuclear architecture, starch accumulation, presence of vacuoles and ribosomal population were studied to characterize sequential stages of microspore embryogenesis and other pathways occurring *in vitro*. *In situ* approaches revealed cell architecture rearrangements during the two pollen developmental programmes, gametophytic and embryogenesis, involving proliferation and differentiation processes, as well as defined changes associated to non-embryogenic pathways followed *in vitro*.

MATERIALS AND METHODS

Plant material and in vitro microspore culture

Brassica napus L. Cv. Topas donor plants were grown under controlled conditions of 18ºC, 80% humidity and a photoperiod of 16 hours light and 8 hours darkness. Isolated microspore culture and embryogenesis induction was performed by a 32ºC heat treatment, as described previously (Custers *et al.* 1994). Microspores cultures at 18ºC were also performed as a system of gametophytic development *in vitro* (Custers *et al.* 1994).

Low temperature processing for light and electron microscopy

Anthers of different bud sizes and *in vitro* cultures containing microspores and microspore-derived structures at specific times (0, 1, 3, 7 …days) were fixed in 4% paraformaldehyde in PBS, overnight, at 4ºC. Isolated microspores were then embedded in gelatine and processed as the anthers, as described before (Seguí-Simarro *et al.* 2003,2005). Samples were dehydrated in methanol by progressive lowering of temperature (*PLT),* and embedded in Lowicryl K4M at –30 ºC under U.V. light (Testillano *et al.* 1995).

For ultrastructural studies, some samples were processed following a more conventional method for electron microscopy: they were fixed in Karnovsky fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.025 M cacodylate, pH 7.3), dehydrated in an ethanol series and embedded in Epon. Ultrathin sections were counterstained by uranyl acetate and lead citrate and observed in a Jeol 1010 EM at 80 kV.

Cytochemical methods

Lowicryl semithin sections (1 μm) were stained with Toluidine blue and observed under bright field for structural analysis. Starch was detected by I_2KI staining on semithin Lowicryl sections and observed under bright field. DAPI staining for DNA was applied to semithin Lowicryl sections and observed under UV light in an epifluorescence microscope (Barany *et al.* 2005).

Immunocytochemistry

Semithin Lowicryl sections were blocked with 5% BSA in PBS (5 min), and incubated with anti-RNA mouse monoclonal antibody (BWR4, Eilat and Fischel 1991, Mena *et al.* 1994) for 1h, and then with anti-mouse IgG conjugated to 10 nm gold particles (Biocell, Cardiff, UK) diluted 1:25 in 1% BSA (45 min). Gold particles were visualized using a silver-enhancing kit (British BioCell International). The sections were finally air-dried, mounted with eukitt and observed under bright field.

RESULTS

In vivo gametophytic development

The *in vivo* gametophytic development of the microspore was monitored by microscopical analysis of selected anthers. The correlation between sequential flower bud lengths and pollen developmental stages was performed (Fig. 1). It constituted the first criterion for handling and selection of specific stages for the microspore culture and for the study of the gametophytic pathway, even though a certain level of asynchrony was observed among buds of the same lengths. Buds from 2.5 to 3.5 mm length usually contained unicellular microspores, larger buds housed bicellular and tricellular pollen. After the release of the tetrade, young microspores exhibited a large and central nucleus, and a dense cytoplasm with no vacuoles (Figs. 1b, b'). At later stages, the microspore developed a large cytoplasmic vacuole which pushed the elongated nucleus to a peripheral location; the thin layer of cytoplasm appeared dense (Figs. 1c, c'). Anti-RNA immunocytochemistry provided a high signal in the cytoplasm of vacuolated microspores (Fig. 1g), indicating an abundant ribosome population. Ultrastructural analysis revealed the cytoplasm of the vacuolated microspore with plastids, mitochondria and other organelles, surrounding the large vacuole (Fig. 1j); the nucleolus showed abundant granular component and small fibrillar centers (Fig. 1j), a typical organization of high biosynthetic activity (Risueño and Medina 1986). After the first pollen mitosis, the small and lens-shaped generative cell was localized attached to the pollen wall (exine) with a very condensed chromatin, as revealed by the DAPI staining (Figs. 1d, d'). In contrast, the larger and rounded vegetative nucleus was in the center and displayed a more decondensed chromatin showing less fluorescence intensity by DAPI (Figs. 1d, d'). The vegetative cytoplasm showed intense anti-RNA labeling (Fig. 1h), both nuclei being free of labeling except for the nucleoli. Controls avoiding the first antibody did not show significant labeling (Fig. 1i). Electron micrographs showed the vegetative and generative cytoplasms containing varoius organelles, granules and vesicles (Fig. 1k). Later on, the generative cell migrated to the center of the pollen grain (Figs. 1e, e') and underwent the second mitosis originating the two sperm cells, which were smaller and with a very condensed chromatin (Figs. 1f, f'). No starch deposits were detected by the Iodide-based cytochemistry in the matured pollen (Fig. 1l).

In vitro gametophytic development

Microspores cultures at 18ºC were performed to follow the gametophytic development *in vitro*. The vacuolate microspores (Fig. 2a) which initiated the culture developed, and structures similar to bicellular (Figs. 2b-d) and tricellular (Fig. 2e) pollen grains were observed after a few (1-3) days. They showed the typical vegetative, generative or sperm nuclei and dense cytoplasms, with no large vacuoles, rich in ribosomes, granules and vesicles. No multicelular structures or embryos were observed. In contrast with the pollen grains developed *in vivo*, an accumulation of starch granules in specific cytoplasmic areas was observed in many of these *in vitro* developed pollen grains, as revealed by the iodide-based cytochemistry (Fig. 2f). At ultrastructural level, numerous and large amyloplasts containing several large starch granules each were observed in the cytoplasm of the *in vitro* developed pollen (Fig. 2g).

In vitro development after the inductive stress treatment

Heat treatment at 32ºC has been revealed as the most efficient inductive treatment to switch the microspore to embryogenesis in *Brassica napus* (Custer *et al.* 1994). In cultures treated at 32ºC for 8 hours, many microspores initiated an embryogenic programme, but other cells of these heat-treated cultures did not switch to the embryogenic pathway and exhibited a structural organization which mimicked that of pollen grains, with a dense cytoplasm rich in ribosomes, organelles, endoplasmic reticulum and vesicles (Fig. 3), with the difference that they contained abundant starch granules (Figs. 3a-d). In many of these cells following a non-embryogenic pathway, a high accumulation of starch was observed concentrated in a concrete cytoplasmic region, between the nuclei and the exine (Fig. 3b), similar to that found in cultures kept at 18ºC where a pathway close to the gametophytic development was followed. In the stress-treated cultures, no bicellular or tricellular pollen were observed among the non-embryogenic structures, indicating that pollen mitosis was blocked in most cases, after the heat treatment.

In heat-treated cultures, the responsive microspores started the embryogenic pathway (Fig. 4) with a symmetric cell division giving rise to two-cell structures (Fig.

4a) one day after the inductive treatment. The resulting two cells and nuclei were similar in size, shape and organization, in contrast with the bicellular pollen originated during the gametophytic development. The cytoplasms appeared dense, with no large vacuoles. No starch deposits or very few were found in the two-cell embryogenic structures (Fig. 4b). Later on, subsequent divisions took place originating multicellular structures, still confined inside the exine (Figs. 4c, d). These cells contained a few starch deposits, as revealed by the iodide-based cytochemistry, which appeared dispersed in the cytoplasms (Fig. 4d); straight walls also characterized the cells of these multicellular structures. These cells displayed large central nuclei with one nucleolus each. An intense anti-RNA labeling was obtained in the cytoplasm of the multicellular structures (Fig. 4e) indicating a high ribosomal population. Ultrastructural analysis showed the cytoplasm rich in organelles, vesicles, endoplasmic reticulum and ribosomes; some amyloplasts containing a few starch grains, as well as small vacuoles appeared homogeneously distributed throughout the cytoplasm (Figs. 4k, l). At later stages, the exine breakdown due to the numerous dividing cells, originating rounded structures or proembryos in which some exine remnants could be observed at the periphery (Figs. 4f, g). They were formed by numerous small polygonal cells in which presence of dispersed starch grains can be found (Fig. 4g). The ultrastructural characteristics of the proembryo cells were similar to that found before the exine breakdown, displaying the typical organization of proliferative cells (Testillano *et al.* 2005). In subsequent developmental stages, globular embryos were observed (Figs. 4h, i) formed by small polygonal cells with large central nuclei and dense cytoplasm rich in RNA, as revealed by anti-RNA immunolabelling (Fig. 4i). At further developmental stages, embryos elongated in some areas giving rise to heart-like embryos (Fig. 4j). A peripheral layer of small, lined-up, isodiametric cells, similar to a protodermis (Fig. 4h, j) could be observed in most of these embryos.

DISCUSSION

In non-heat-treated cultures, the microspore follows a gametophytic-like pathway with a differential starch accumulation

In vitro cultures of *Brassica napus* microspores have been developed to induce the switch to the sporophytic pathway in a high frequency by heat treatments (Pechan and Keller 1988, Telmer *et al.* 1992). A decrease in the temperature of the culture has been reported to lead to a microspore development similar to the gametophytic pathway (Custers *et al.* 1994) but no a detailed analysis of the subcellular organization of these microspores has been pursued until the present work.

In the scheme of figure 5 the different developmental pathways followed by the microspore *in vivo* and *in vitro* are summarized. When compared with the *in vivo* gametophytic development, the *in vitro* cultured microspore mimics many cellular features occurring *in vivo*, i.e. the reabsortion of the large vacuole, the first and second pollen mitosis, the cytoplasm dense and rich in vesicles, the organelles and ribosomes, the cell sizes and shapes, and also the chromatin condensation pattern of the vegetative, generative and sperm cell nuclei. The results presented here illustrated that the main difference between the gametophytic development *in vivo* and *in vitro* was a high accumulation of starch which only takes place *in vitro*, being concentrated in a specific cytoplasmic region, between the nucleus and the exine.

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). In several species, the carbohydrate reserves of the mature pollen are not in the form of starch granules, but in the form of cytoplasmic polysaccharides and specially sucrose (Franchi *et al.* 1996, Pacini 1996). In microspore cultures of *Brassica napus* under non-inductive conditions, the accumulation of starch accompanied the progression of the gametophytic pathway and the pollen divisions, resulting in bicellular and tricellular pollens with high starch content, in comparison with the *in vivo* matured pollens, which do not show starch accumulations. The cytoplasmic localization of the starch grains in only one half of the

pollen grain constitutes a specific feature of the *in vitro* development of the microspore, which seems to be independent of the developmental pathway followed.

In heat-treated cultures, the microspore can follow embryogenic and non-embryogenic pathways which differ in subcellular organization, starch distribution and accumulation

The structural pattern of development of microspore-derived embryos is still poorly understood. In rapessed, a comparison with the formation of the zygotic embryo has been described, the study being mainly focused to the structural patterns of tissue and meristems formation (Yeung *et al.* 1996). Reports on the ultrastructural analysis of the first embryogenic division of *Brassica* microspores have characterized the symmetric division and cell wall formation after induction (Zaki and Dickinson 1990, 1991). The results presented here showed that the initial proliferation and the formation of multicellular structures and proembryos do not follow a regular and defined pattern of divisions, as the zygotic embryogenesis (Raghavan 2000, Yeung *et al.*,. 1996), as it has been also shown in other species (Testillano *et al.* 2002, Barany *et al.* 2005).

In this work, the presence of starch during the embryogenic development has been analyzed by the iodide-based cytochemistry, the results indicating its presence in multicellular proembryos, still surrounded by the exine. The subcellular distribution of starch granules was homogeneous in the cytoplasms of cell proembryos. Early starch accumulation in microspore-derived proembryos still surrounded by the exine has been described in some reports on cereals (Indrianto *et al.* 2001, Testillano *et al.,* 2002), showing an heterogeneous distribution in the proembryos which was suggested to be in relation to the existence of different cell domains and fates (Testillano *et al.* 2002, Ramírez *et al.* 2001). In pepper, starch has been found in larger microspore proembryos, after exine breakdown, which was suggested to be associated to early differentiation events (Barany *et al.* 2005).

At further developmental stages, the differentiation of a protodermis in globular embryos and the ulterior development of heart-like embryos mimic the main events of zygotic embryo formation (Raghavan 200, Yeung *et al.* 1996).

Together with the embryogenic development, other non-embryogenic pathways followed by the non-induced microspores, which have not been described until now, occurred after the inductive treatment. They displayed structural features similar to those found in non-heat treated cultures, especially concerning the organization of the cytoplasms and the specific starch accumulation, mainly in one cytoplasmic region. After the stress treatment, changes in expression of heat shock proteins and MAP kinases have been reported in *Brassica napus* microspore cultures (Cordewener *et al.* 1995, Segui-Simarro *et al.* 2003, 2005). These changes were also found in non-embryogenic microspores, but in a different level, suggesting that those molecules could have a role not only in the response to the stress but also in the initiation of the embryogenic pathway (Segui-Simarro *et al.* 2003, 2005). The non-embryogenic pathway followed in heat-treated cultures showed some similarities with the gametophytic-like pathway occurring *in vitro* under non-inductive conditions, but pollen mitosis were only rarely observed in heat-treated cultures. Results presented here suggest that the gametophytic programme is not totally stopped by the stress and the microspores which do not switch to the sporophytic pathway can express, in a limited way, many of the metabolic and structural events of the pollen differentiation, with the important exception of the asymmetric mitosis.

The presence of abundant starch grains accumulated in a half of the cytoplasm between the nucleus and the exine, seems to be a specific feature of the *in vitro* non-embryogenic development of the microspore, since it occurs under both, inductive and non-inductive conditions. This fact could also be interpreted as an intrinsic effect of the culture conditions which involve a medium rich in sugars. Further work comparing different culture conditions in several systems will shed light to this question. Polarity is an inherent feature of most eukaryotic cells whose establishment and control mechanisms are far from known (Baluska *et al.* 2003, Samaj *et al.* 2004). The non-embryogenic *in vitro* pathways followed by the microspore, with the starch accumulated in a specific cytoplasmic area, could constitute a convenient system to analyze questions related to the cell polarity establishment and growth.

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FIGURE LEGENDS

Figure 1: Microspore gametophytic development *in vivo*.

a: *Brassica* flower buds at sequential developmental stages containing microspores at stages from young microspore till mature tricellular pollen. **b-f, b'-f':** Cell architecture at different stages of gametophytic development, b-f: toluidine blue staining, b'-f': DAPI staining for DNA. b, b': Young microspore, c, c': vacuolated microspore, d, d': young bicellular pollen, e, e': mid bicellular pollen, f, f': mature tricellular pollen. **g-i:** Anti-RNA immunolocalization: vacuolated microspore (g), bicellular pollen (h), and control experiment (i). **j, k:** Ultrastructure of the vacuolated microspore (j) and the young bicellular pollen (k). **l:** I_2K cytochemistry for starch in mature tricellular pollen, no staining is observed. Ct: cytoplasms, V: vacuole, N: nucleus, Nu: nucleolus, Ex: exine, GC: generative cell, VC: vegetative cell, W: cell wall of generative cell. Bars in a: 5 mm, in b-i, l: 5 μm, and in j, k: 2 μm.

Figure 2: Microspore gametophytic development *in vitro,* at 18ºC.

a-e: Cell architecture at sequential stages of the culture: vacuolated microspore (a), young bicellular (b), mid bicellular (c), late bicellular (d) and tricellular (e). **f:** I_2K cytochemistry for starch in cultures of 5-6 days containing late bicellular and tricellular pollen. **g:** Ultrastructural organization of a late bicellular pollen culture *in vitro*. Arrow: generative cell, double arrow: sperm cells, Ex: exine, S: starch deposits, VN: vegetative nucleus, GN: generative nucleus. Bars in a-f: 10 μm, and in g: 2 μm.

Figure 3: Microspore gametophytic-like development *in vitro*, after 32ºC treatment.

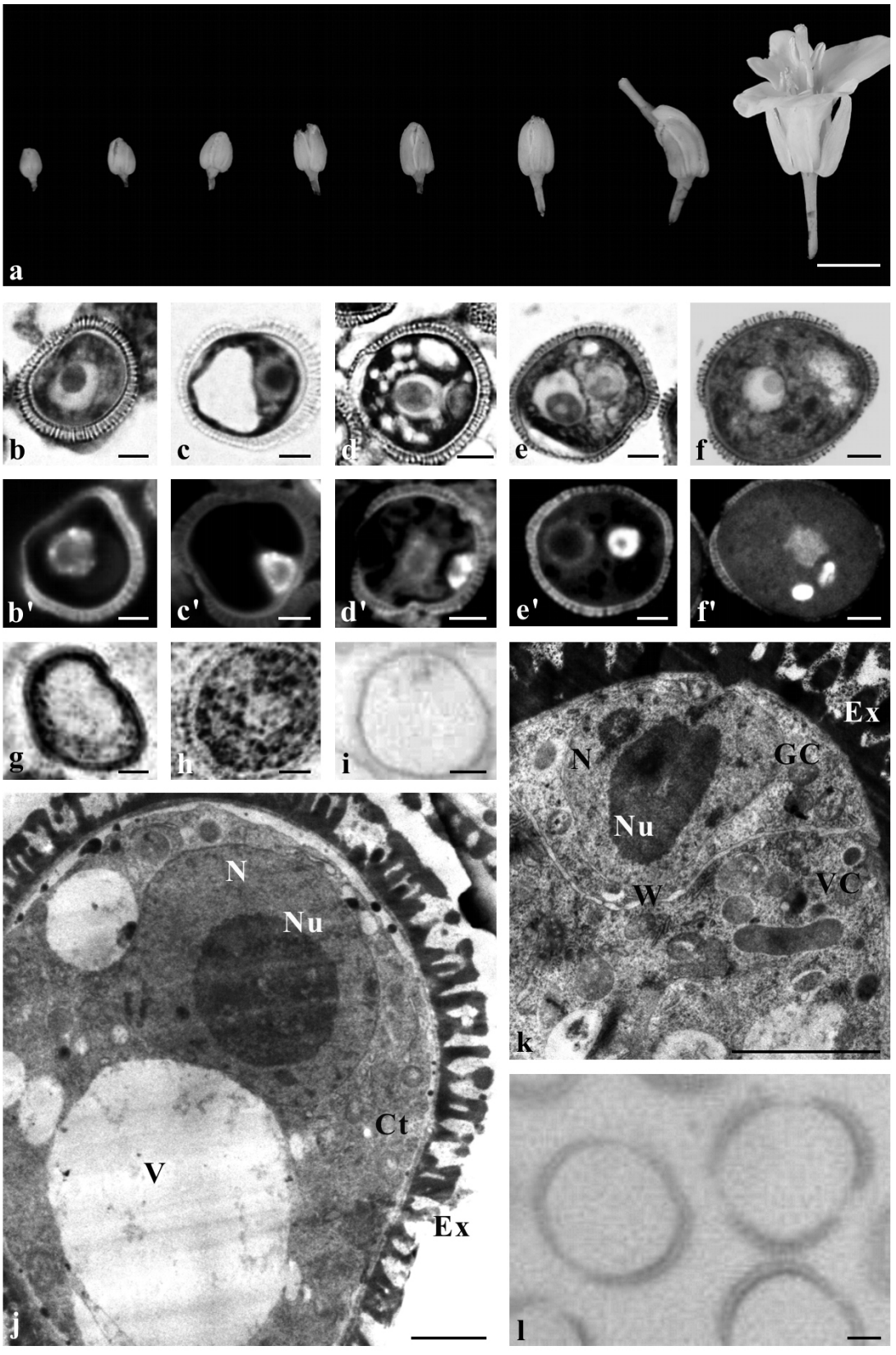
a-d: Cell architecture, after toluidine blue staining (a, c) and starch localization, revealed by the I_2K cytochemistry (b, d). **e:** electron micrograph showing the ultrastructure of a microspore developed *in vitro* at 32ºC similar to that showed in c-d. Ex: exine, N: nucleus, Nu: nucleolus, Ct: cytoplasm. Bars correspond to 10 μm.

Figure 4: Microspore embryogenic development *in vitro,* after 32ºC treatment.

a-d: First developmental stages as seen after toluidine blue staining (a, c) and I_2K cytochemistry for starch (b, d). a, b: two-cell structure, c, d: Multicellular proembryo

still confined by the exine. **e-j:** Multicellular proembryos after exine bearkdown (e-g), globular (h, i) and heart-shaped (j) microspore-derived embryos. e, i: Anti-RNA inmunolocalization, g: starch cytochemistry**. h, i:** Ultrastructural organization of multicelular proembryos at the stage of exine rupture. Ex: exine, Ct: cytoplasm, W: cell wall. Bars in a-g: 10 μm, in h-j: 50 μm, and in k, l: 2 μm.

Figure 5: Scheme summarizing the microspore developmental pathways *in vivo* and *in vitro*. Grey rounded structures represent the nucleus of the microspore, vegetative nucleus of pollen and proembryo nuclei. Grey ellipsoid structures represent the generative and sperm nuclei in bicellular and tricellular pollen respectively. Black spots represent starch deposits.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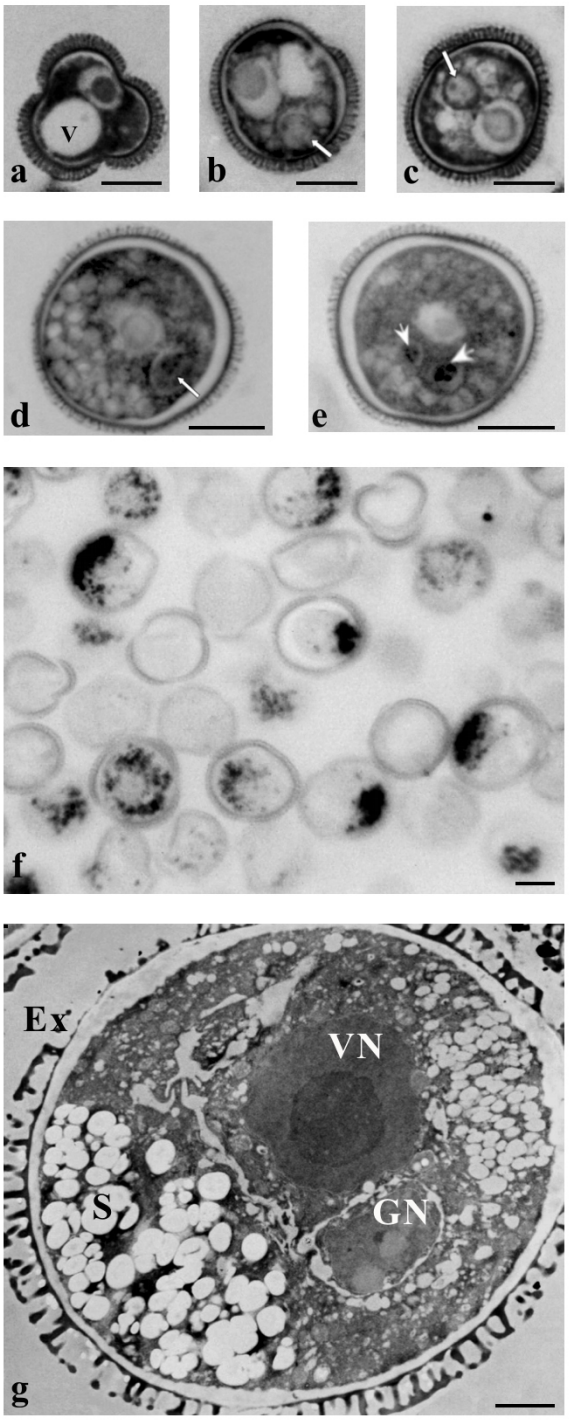
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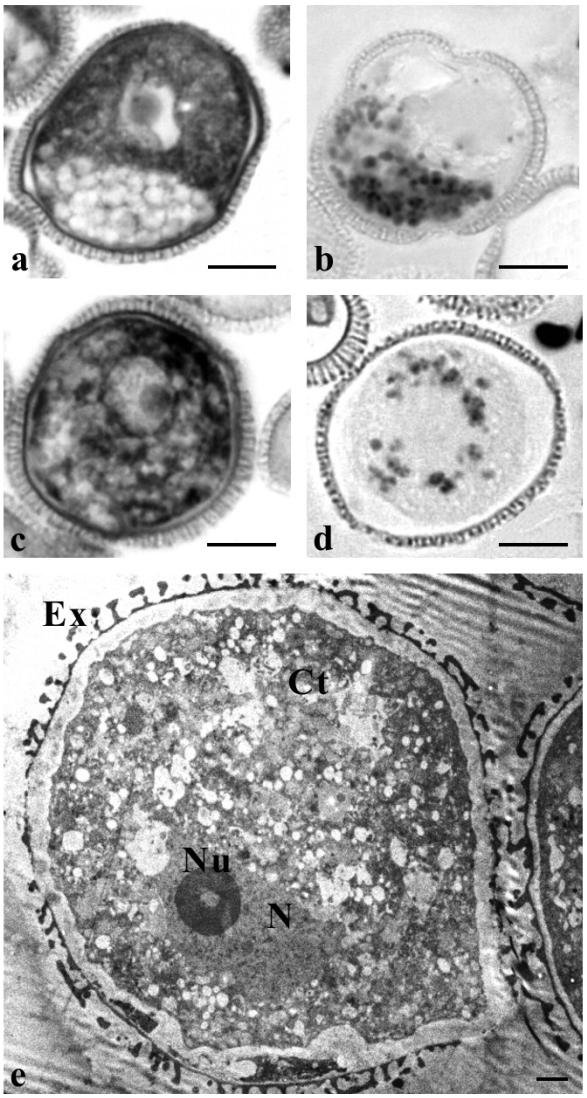
a
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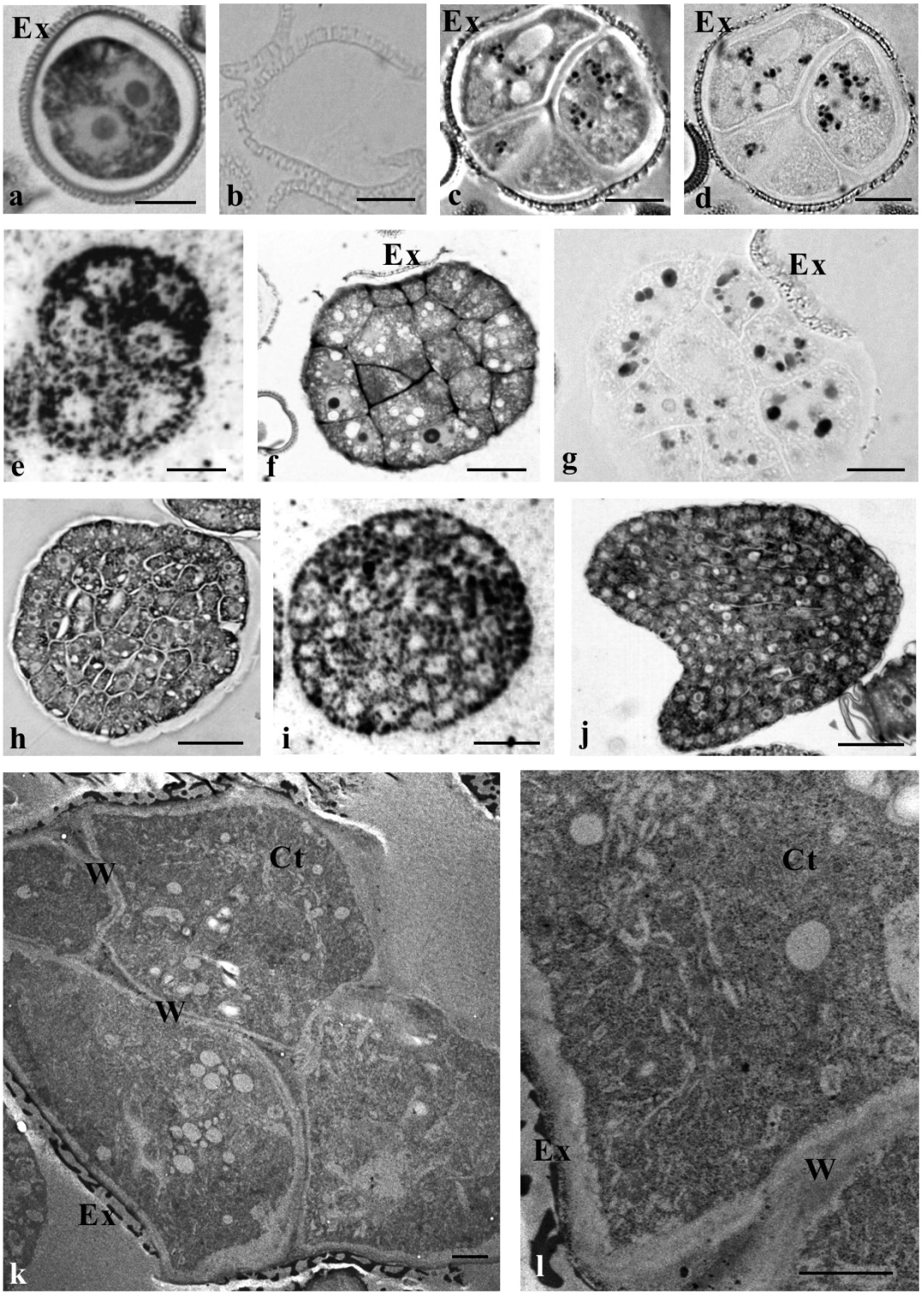
Ex
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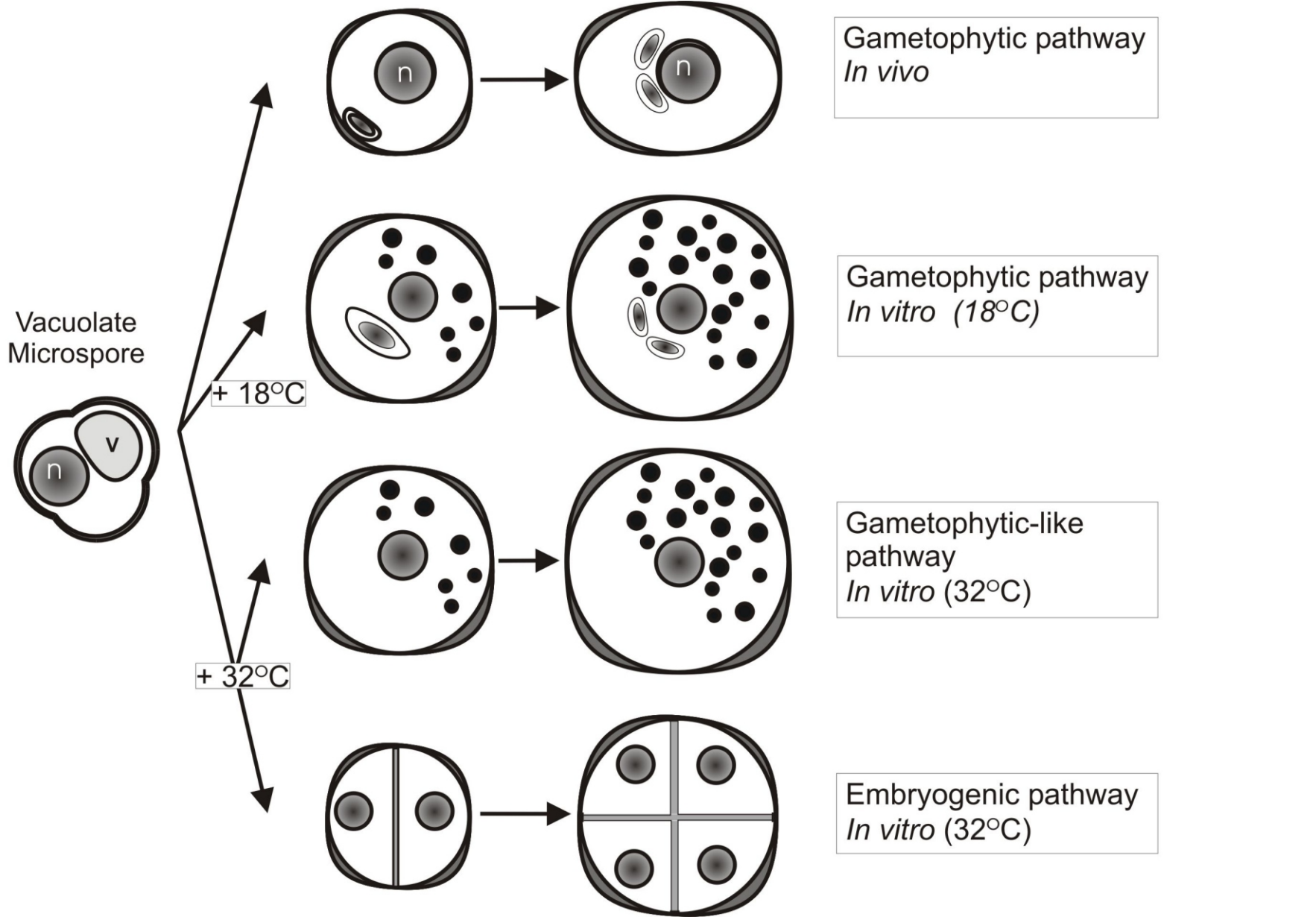
Vacuolate Microspore
n
v
+ 18°C
+ 32°C
n
n
Gametophytic pathway
In vivo
Gametophytic pathway
In vitro (18°C)
Gametophytic-like pathway
In vitro (32°C)
Embryogenic pathway
In vitro (32°C)