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FIRST EMBRYOGENIC STAGES OF *CITRUS* MICROSPORE-DERIVED EMBRYOS

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This work is the first characterization at cellular and subcellular level of the main cellular events occurring in the first stages of microspore embryogenesis. Microspore embryogenesis was induced in two varieties of *Citrus clementina* (Nules and Monreal). The results showed that one of the most responsive stages for embryogenesis was the vacuolate microspore. Microscopic analysis revealed specific features of the young proembryos still surrounded by the exine: large nuclei, clear areas in the cytoplasm, starch accumulation, and an increase in the thickness of the wall under the exine. Immunogold labelling with JIM 5 antibody showed a high amount of non-esterified pectins in the surrounding cell wall. After exine rupture, different cell types were detected in late proembryos. As embryogenesis proceeded, the normal pattern of development was observed, including heart-shape, torpedo and cotyledonar embryos.

Key words: *Citrus*, haploids, microspore embryogenesis, ultrastructure, cytochemistry.

INTRODUCTION

Haploid plants are of great interest to breeders and geneticists, mainly for their potential in mutation research, genetic analysis and production of inbred lines, particularly in fruit trees which are characterized by a long reproductive cycle, a high degree of heterozygosity, and often self-incompatibility. Haploids are obtained by in vitro induction of microspore embryogenesis, normally achieved with different stress treatments.

In order to make microspore embryogenesis a reliable tool in *Citrus* breeding, it is necessary to improve the induction response (the frequency of microspores forming embryos) and also to increase the number of responsive genotypes. Anther culture technique has been used to obtain haploid and double-haploid embryogenic calli, plantlets and in vivo grafted plants in *C. clementina* Hort. ex Tan. (Germanà and Reforgiato-Recupero, 1997). At present there are no extensive studies in *Citrus* focused on characterizing this process at the cellular level. This type of study could provide new insights into the key processes involved in the switch and progression of the microspore to embryogenesis. It should lead to high efficiency of embryogenesis induction, as has been reported in herbaceous and woody plants (González-Melendi et al., 1996; Ramírez et al., 2001a).

This paper reports the first cellular and ultrastructural characterization of the early stages of microspore embryogenesis in *Citrus*.

MATERIALS AND METHODS

PLANT MATERIAL AND IN VITRO ANTHER CULTURE FOR MICROSPORE EMBRYOGENESIS

Flower buds were collected in March 2001 from 25-year-old trees of *C. clementina* Hort. Ex Tan, cv Nules and cv Monreal. The stage of pollen develop-

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ment was checked in one anther per bud by DAPI staining in squashed anthers. Buds with microspores at the late uninucleate stage were selected. After pretreatment at 4˚C for 14 days, the buds were sterilized. Anthers were cultured in vitro as previously described (Germanà et al., 1991; 1994). Embryogenic anthers were selected and fixed for microscopic analysis at different culture times (at the beginning, after pretreatment, and through five months in culture).

CRYOPROCESSING FOR LIGHT AND ELECTRON MICROSCOPY

Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.3, overnight at 4˚C. After washing in PBS, samples were dehydrated through a methanol series by progressive lowering of temperature (PLT) from 0˚C to -30˚C. Then the samples were infiltrated and embedded in Lowicryl K4M resin at -30˚C under UV irradiation. Semithin sections were made and contrasted with toluidine blue for light microscopy. Ultrathin sections were made for ultrastructural and immunolocalization studies at the electron microscopy level.

CYTOCHEMICAL STUDIES FOR DNA AND STARCH AND IMMUNOGOLD LABELLING

Lowicryl semithin sections were stained with DAPI (10 µg/ml) for 5 min at 45˚C to stain the DNA. Staining with potassium iodide (2%) for 10 min at 45˚C was done to preferentially stain starch.

Immunogold labelling was performed on Lowicryl ultrathin sections as described previously (González-Melendi et al., 1996). Sections were incubated with JIM 5 rat monoclonal antibody recognizing non-esterified pectins (Casero and Knox, 1995) and anti-rat IgG secondary antibody conjugated to 10 nm gold particles.

RESULTS

Anthers fixed from the tree displayed a great number of vacuolate microspores inside the pollen sac (Fig. 1a,b). They exhibited a large cytoplasmic vacuole which pushed the nucleus towards the periphery. After 14 days of cold pretreatment, the survival rate of the microspores was high, and most of them displayed cellular features of young bicellular pollen, with the generative cell located at the periphery (arrow in Fig. 1c), the vegetative nucleus in the center, and dense cytoplasm (Fig. 1c). Some microspores showed unusual cellular features in comparison with previous and subsequent stages: a large peripheral nucleus and some clear areas in the cytoplasm (arrowhead in Fig. 1c). After two months in culture, some microspores changed their developmental program and multicellular proembryos still surrounded by the exine appeared; they showed several nuclei of similar sizes, shapes and chromatin condensation patterns, as revealed by DAPI staining (Fig. 1). The IIK cytochemical assay revealed that these proembryos accumulated starch granules in the cytoplasm (Fig. 1e).

At the electron microscopy level, young microspore-derived proembryos showed a characteristic ultrastructure with several clear areas in the cytoplasm. A thick cell wall of medium electron density was present between the exine and plasmalemma (Fig. 1f). Immunoelectron microscopy with JIM5 antibody for non-esterified pectins showed a high density of gold particles unevenly distributed over the cell wall surrounding the proembryo (Fig. 1g). Gold particles were not observed in unexpected cell compartments.

At three months in culture medium, as embryogenesis proceeded, late microspore proembryos appeared after the breakdown of the exine. They formed as round proliferative masses in the interior of the anther in the pollinic sac (Fig. 2a). These proembryos showed two different cellular types: small inner cells, polygonal-shaped, having dense cytoplasm and large nuclei with a decondensed chromatin pattern, resembling proliferative cells ("Ic" in Fig. 2b); larger outer cells at the periphery of these proembryos, showing clear cytoplasm with big vacuoles ("Oc" in Fig. 2b). These proembryos seemed to follow a developmental pathway similar to that of zygotic embryogenesis, producing globular, heart, torpedo and cotyledonar embryos. At the heart-shaped stage, microspore-derived embryos showed a peripheral layer of cells, similar to protodermis (Fig. 2e,f). Cells of heart-shaped embryos exhibited highly vacuolate cytoplasm and ellipsoid nuclei with a specific pattern of chromatin condensation, as revealed by DAPI staining, with highly fluorescent spots at the nuclear periphery (Fig. 2c,d), indicating that condensed chromatin was localized in this area. At the interior of the heart-shape embryos, some small areas with large and rounded nuclei were observed (Fig. 2c,d), probably corresponding to areas of proliferating cells. Later in the developmental process, cotyledonar embryos emerged from the anther (Fig. 2g) and continued the developmental process to give rise to a plant.

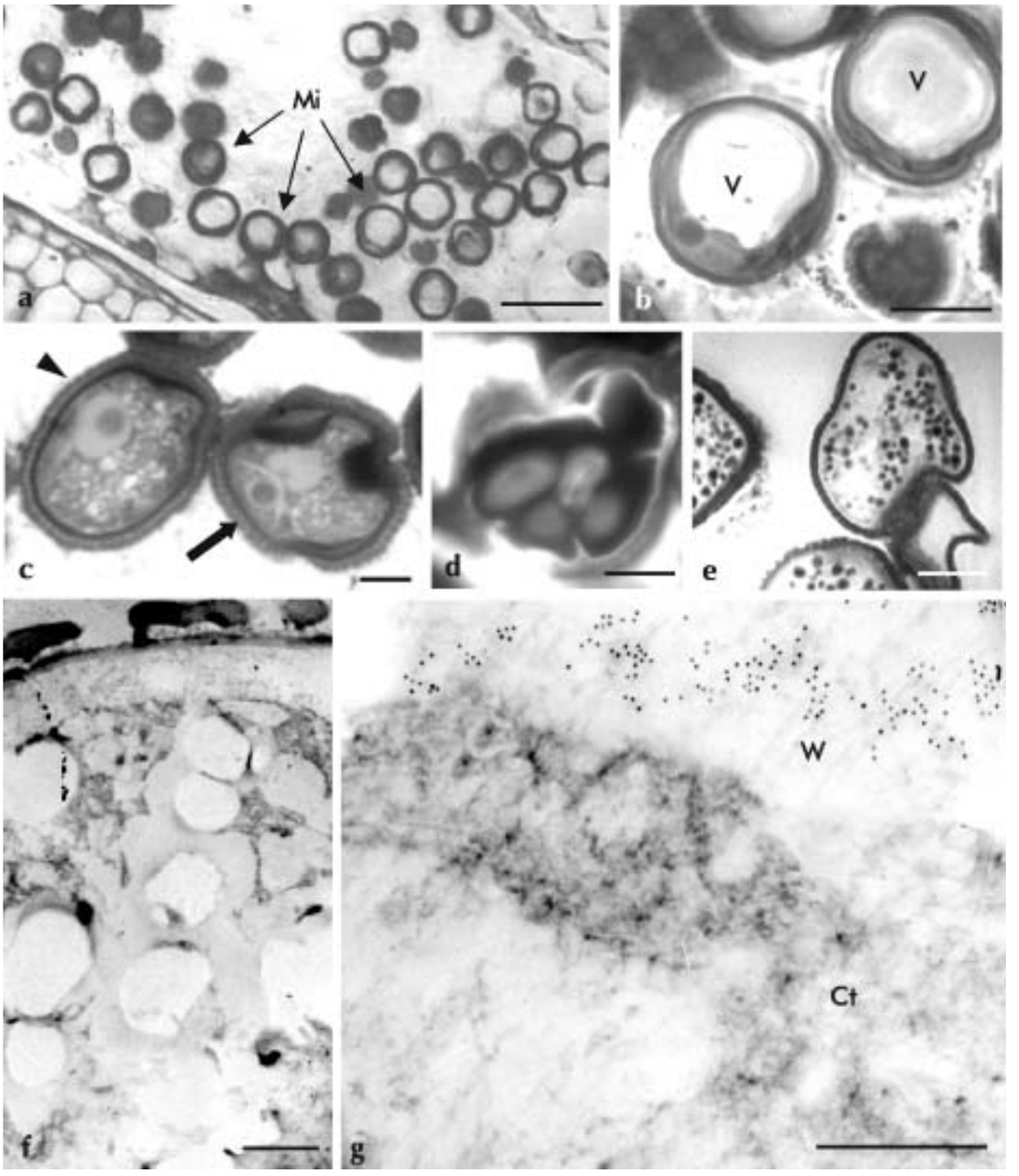
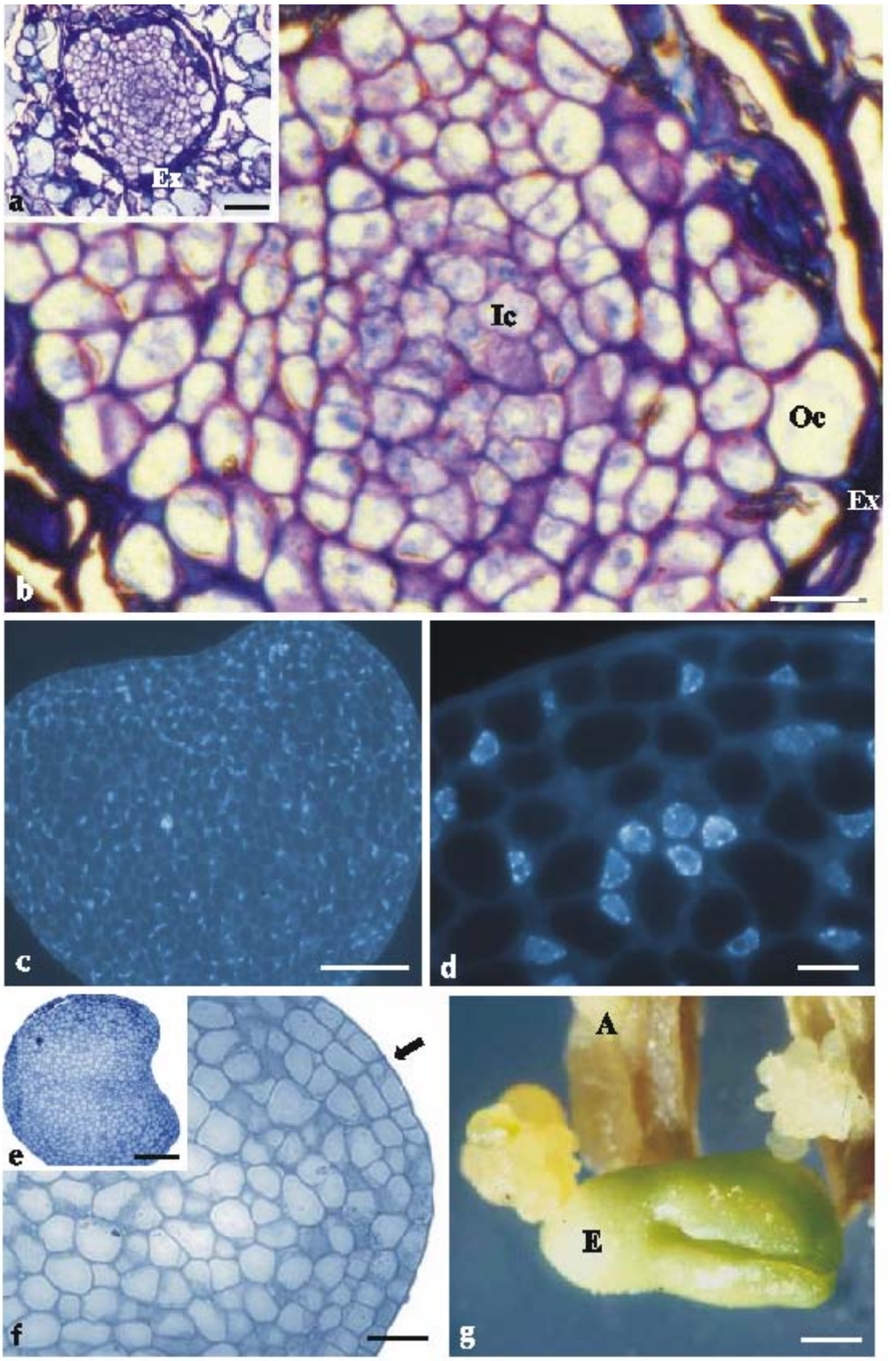


Fig. 1. (**a**) Anther at the beginning of culture. Lowicryl semithin section, toluidine blue staining, (**b**) Vacuolate microspores at the interior of the pollen sac, (**c**) Microspores after pretreatment. Lowicryl semithin section, toluidine blue staining, (**d**) Young proembryos still surrounded by the exine, DAPI staining, (**e**) Young proembryo cytochemistry with IIK revealing starch granules, (**f**) Ultrastructure of young proembryo, (**g**) Immunogold labelling for non-esterified pectins with JIM5 antibody, showing high labelling in the cell wall (W) surrounding the proembryo. Ct – cytoplasm; Mi – microspores; V – vacuoles. Bars in (a) = 50 μm, in (b) = 10 μm, in (c–e) = 5 μm, in (f) = 2 μm, and in (g) = 0.5 μm.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DISCUSSION

This work reports for the first time the main changes in cellular organization during the first stages of microspore embryogenesis in *Citrus clementina*. There are only a few previous studies on the morphology of *Citrus* anthers after several days in culture (Hidaka et al., 1982; Hidaka and Omura, 1989). As far as we know, this is the first time that the sequence of developmental events of microspore embryogenesis in *Citrus* has been reported using ultrastructural, cytochemical and immunocytochemical approaches. Selection of appropriate processing methods for good structural and immunological preservation is necessary for this work, because cellular processing is more difficult in woody species (Ramírez et al., 2001b). With this study we have established the conditions for adequate sample processing to obtain good fixation and preservation of *Citrus* anther structure, and the best conditions for in situ and immunolocalization studies.

Observations of numerous vacuolate microspores in samples at different culture times, that is, anthers fixed directly after extraction from the tree, after pretreatment, and after days and months in culture, suggest that the vacuolate microspore could be the most responsive stage for embryogenesis induction, as reported for herbaceous species (González-Melendi et al., 1995) and for a tree, *Quercus suber* (Ramírez et al., 2001b; Ramírez et al., 2002a). The numerous microspores found in anthers after the pretreatment, with an organization typical of young bicellular pollen grains, indicate that in *Citrus* the majority of the microspores survive and continue their developmental program. The presence of some microspores with different features (big peripheral nuclei and clear areas in the cytoplasm) not typical of the gametophytic pathway could indicate that after pretreatment these changes in cellular organization accompany the switch of the developmental program towards embryogenesis (Testillano et al., 2000; Ramírez et al., 2001a).

The first multicellular structures appeared later on. These proembryos showed large nuclei of similar size and with similar features, suggesting that they were cells in proliferation (Testillano et al., 2000). A special characteristic, similar to the proembryos of other species, was the presence of starch granules in their cytoplasm. This feature has not been described previously; only cytoplasm with clear areas was reported in embryogenic microspores of *Citrus* (Hidaka and Omura, 1989). Another feature of these proembryos was the thickness of the wall under the exine, which appeared after the induction phase. An increase in wall thickness has been described in microspore proembryos of other species (González-Melendi et al., 1995). The functional significance of these structures is not yet known, but would fit in with the presence of other wall components in embryogenic structures (unpublished results). In the cell wall of these proembryos we detected high density of non-esterified pectins (with less than 40% methyl esterification) under the exine. A high proportion of esterified versus non-esterified pectins was reported to be characteristic of the wall of proliferative cells at early stages (Ramírez et al., 2002b). High amounts of non-esterified pectins found in *Citrus* were also found in *Quercus* microspore proembryos (Ramírez et al., 2002a), and could also be related to the structural strength needed in the cell wall of woody species. This feature would be a good marker for the first pollen embryogenic stages, since the presence of other arabinogalactoproteins has been reported in the walls of cells committed to forming an embryo in somatic embryogenesis systems (McCabe et al., 1997).

As embryogenesis proceeds, embryo stages appear similar to those described for zygotic embryogenesis – heart, torpedo and cotyledonar embryos – indicating that microspore and zygotic embryogenesis follow analogous developmental programs (Bueno et al., 2002; Ramrez et al., 2002a).

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Fig. 2. (**a**) Late proembryo located in interior of anther. Fragments of broken exine are still at its periphery. Lowicryl semithin section, toluidine blue staining, (**b**) Late proembryo at higher magnification, showing two different cellular types in the inner (Ic) and outer (Oc) areas, (**c**) Heart-shaped embryo, DAPI staining for DNA revealing certain areas with groups of large nuclei, (**d**) Higher magnification of one of these areas, (**e**) Heart-shaped embryo. Lowicryl semithin section, toluidine blue staining, (**f**) Higher magnification of heart-shaped embryo showing the protodermis at its periphery, (**g**) Cotyledonar embryo emerging from an anther. Ex – exine; Ic – inner cells; Oc – outer cells; A – anther; E – embryo. Bars in (a) = 25 μm, in (b) = 15 μm, in (c) = 50 μm, in (d) = 4 μm, in (e) = 25 μm, in (f) = 5 μm, and in (g) = 1 mm.

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