

# Young microspore-derived maize embryos show two domains with defined features also present in zygotic embryogenesis

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**ABSTRACT** In this work we report the existence of two domains in early microspore maize proembryos displaying similar features of zygotic embryogenesis. The large or so-called endosperm-like domain exhibits specific features: coenocytic organisation, synchronous mitosis, vacuolated cytoplasm, starch granules, incomplete walls containing callose and differential tubuline organisation. The small or embryo-like domain displays small polygonal uninucleate cells with typical organisation of proliferating cells. The structural organisation and the subcellular localization of specific cytoplasmic and cell wall components (starch, tubuline and callose) in both proembryo domains have been determined by using specific cytochemical and immunocytochemical methods. Four morphological types of proembryos containing both domains have been characterised. Taking into account their relative size, the high asynchrony of the culture and the homologies between structural features of endosperm-like domain and zygotic endosperm development, they could represent different stages in microspore embryogenesis development. The *ZmAE1* and *ZmAE3* genes expressed in the Embryo Surrounding Region of the endosperm during zygotic embryogenesis (Magnard *et al.*, 2000), were revealed to be expressed in early microspore proembryos by *in situ* hybridization at light and electron microscopy levels. This data supports the existence of an endosperm-like function during early microspore embryogenesis and provides new insights into the onset of microspore embryogenesis in maize, and its parallelism with zygotic embryogenesis.

**KEY WORDS:** *microspore embryogenesis, maize, endosperm, immunocytochemistry, in situ hybridization*

## Introduction

In many plant species it is possible to experimentally deviate pollen development towards an embryogenic pathway by specific stress factors, a process referred to as microspore embryogenesis (Guha and Maheshwari, 1966; Nitsch and Ohyama, 1971; Guha and Maheshwari, 1964; Nitsch and Norreel, 1973; Reynolds, 1997; Touraev *et al.*, 1997). The initial phase of microspore embryogenesis consists of repeated divisions of the induced microspores to give rise to proembryo structures enclosed in the original wall of the former microspores, the exine. After the breakdown of the exine, microspore proembryos develop into haploid embryos and plantlets.

Despite the fact that microspore embryogenesis has been experimentally induced in many plant species, the available data relating to the process is still scarce both at cellular and molecular levels. Cellular events taking place in the first stages after embryogenesis induction can inform in which way the immature male gametophyte, the microspore, changes its behaviour to lead to embryo formation,

and if there are homologies between the first events in zygotic embryogenesis (Domenech *et al.*, 1998; Ramirez *et al.*, 2001b)

Morphological and ultrastructural studies of early pollen embryogenesis are scarce, and many of them deal with dicot species, like *Brassica* and tobacco (Reynolds, 1990; Zaki and Dickinson, 1990; González-Melendi *et al.*, 1995; González-Melendi *et al.*, 1996; Testillano *et al.*, 1996; 2000; Straatman and Schel, 1997; Coronado *et al.*, 2002). In monocots, cellular studies are less numerous; barley is the most responsive to pollen embryogenesis, being analyzed in most cellular and ultrastructural studies of cereal microspore embryogenesis (Dunwell and Sunderland, 1975; Huang, 1986; Kumlehn and Lörz, 1999; Ramirez *et al.*, 2001a). Very little structural data is available on early maize microspore embryogenesis (Barnabas *et al.*, 1987; Góralski *et al.*, 1999; Magnard *et al.*, 2000). The very low response of this species to induction has made microscopical studies

*Abbreviations used in this paper:* DAPI, 4,6-diamidino-2 phenyl-indole; ISH, *in situ* hybridization.

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difficult. The setting up and improvement of a suitable procedure for embryogenesis induction in isolated microspores of maize (Gaillard *et al.*, 1991) and also the development of a method to obtain enriched fractions of very young proembryos in the early steps of the culture (Magnard *et al.*, 2000) have made it possible to perform a more detailed ultrastructural and cytochemical study of these structures, which is reported in this work.

Further development of haploid embryos resembles the normal zygotic embryogenesis pathway in monocot species, by forming globular, transitional, and coleoptilar embryos (Randolph, 1936; Matthys-Rochon *et al.*, 1998). But very little information is provided on the parallelism between both embryogenic programs at their initial process (Magnard *et al.*, 2000) and the mechanism involved. How the development of embryogenic microspores into embryos correlates to zygotic embryo and endosperm development, and how fertilisation, embryo and endosperm development can be reproduced *in vitro* are still open questions (Kranz and Lorz, 1993; Kranz and Kumlehn, 1999).

Two new embryogenesis-specific genes, *ZmAE1* and *ZmAE3*, have recently been isolated in maize; they did not show any expression during pollen development or in other vegetative tissues (Magnard *et al.*, 2000). They were isolated by mRNA differential display from 5/7 day proembryos and showed specific expression, not only at early microspore embryogenesis but also in the first stages of zygotic embryogenesis (Magnard *et al.*, 2000).

This work is addressing some of these questions by analyzing the presence of specific subcellular features and the expression of the mentioned two embryogenesis-specific genes in both developmental programs by specific cytochemical, immunocytochemical and *in situ* hybridization methods.

## Results

### Cellular Organization during the First Steps of Microspore Embryogenesis

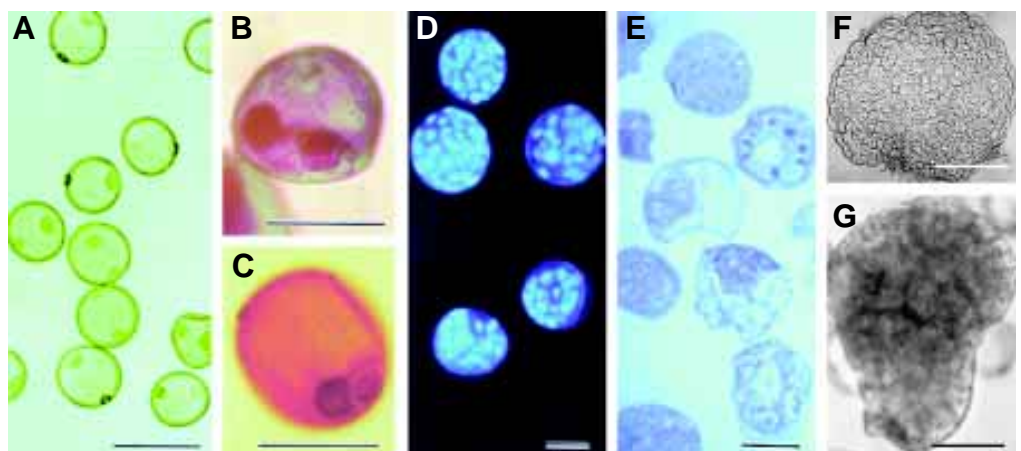
To perform the cellular study of early microspore embryos, enriched fractions of 5/7 day-old proembryos were used, by following the reported inductive and culture procedures (Gaillard

*et al.*, 1991) and the reported method for obtaining samples enriched in young microspore proembryos (Magnard *et al.*, 2000). Pretreatment of the tassels at 7°C for 14 days was done as an inductive procedure for embryogenesis. Microspores were then isolated as reported (Gaillard *et al.*, 1991) and cultured in liquid medium. Just after isolation most of the microspores were shown to be vacuolate, displaying the nucleus at the periphery (Fig. 1A). Some bicellular pollen grains were also observed at the beginning of the embryogenic culture. Soon after plating, ranging from some hours to a few days after the culture started, some structures with two nuclei of similar size and shape could be distinguished (Fig. 1B). These binucleate structures did not have the organization of the gametophytic bicellular pollen, in which generative and vegetative nuclei are different and clearly recognized (Fig. 1C).

The yield of embryogenic induction was low, even though the best responsive genotype in maize was chosen (Gaillard *et al.*, 1991). Five to seven days after plating, multicellular structures appeared in the culture together with many dead cells. Selection of the proembryos could be performed by filtration and centrifugation in a Percoll gradient, as already reported (Magnard *et al.*, 2000). The fraction obtained was enriched with 5/7 day-old proembryos, which after DAPI staining showed around 30-50 nuclei (Fig. 1D) still enclosed in the former microspore wall, the exine. This 5/7 day-old proembryos fraction was selected for the microscopical study of early microspore embryogenesis. Semithin sections stained with toluidin blue showed that these proembryos displayed different morphological organizations (Fig. 1E), some of them with a central vacuole, others with an homogeneous structure, and numerous showing two regions or domains with different staining density, a dark domain and a lighter domain (Fig. 1E). The size of each region is variable, which could reflect the effect of different cutting planes of similar structures.

After more days, e.g. at 10 days after plating, the bursting of the pollen wall was observed in various proembryos, together with others in which the exine was still intact. This fact clearly illustrated the asynchrony of the culture. At day 12, most of the proembryos had completely broken their pollen wall and two main structures could be seen: cellular masses with a elongated protrusion

**Fig. 1. Progression of microspore embryogenesis culture monitored by light microscopy (A)** Microspores after isolation and plating under inverted microscope. Most of them are vacuolate microspores and exhibit one nucleus at the cell periphery; the pollen pore/aperture appears as a dark spot in many microspores. **(B)** A cell with two nuclei, observed some hours after plating in embryogenic cultures. Alexander staining in squash. Both nuclei are similar in size and shape and are localized at the periphery. **(C)** Early bicellular pollen during gametophytic development *in vivo*. Alexander staining in squash. Both nuclei were localized in a pole of the pollen grain and showed some differences between



them: the vegetative is bigger in size and the generative appears smaller and surrounded by a thin cell wall attached to the pollen wall. **(D,E)** Purified 5 day-old proembryos after DAPI staining in squash (D) and toluidine blue staining in 1 µm sections (E). Multicellular structures showed around 30-50 nuclei by DAPI. In section, the different morphologies can be distinguished; many of them exhibit two regions with different staining properties, one clear and another more dense. **(F,G)** Proembryo structures after 14 days in culture. The original pollen wall had broken and two main structures were observed: cellular masses with bulged protrusions (F) and rounded clusters of cells (G). Bars: A,D,E,F,G, 50 µm; B,C, 25 µm.

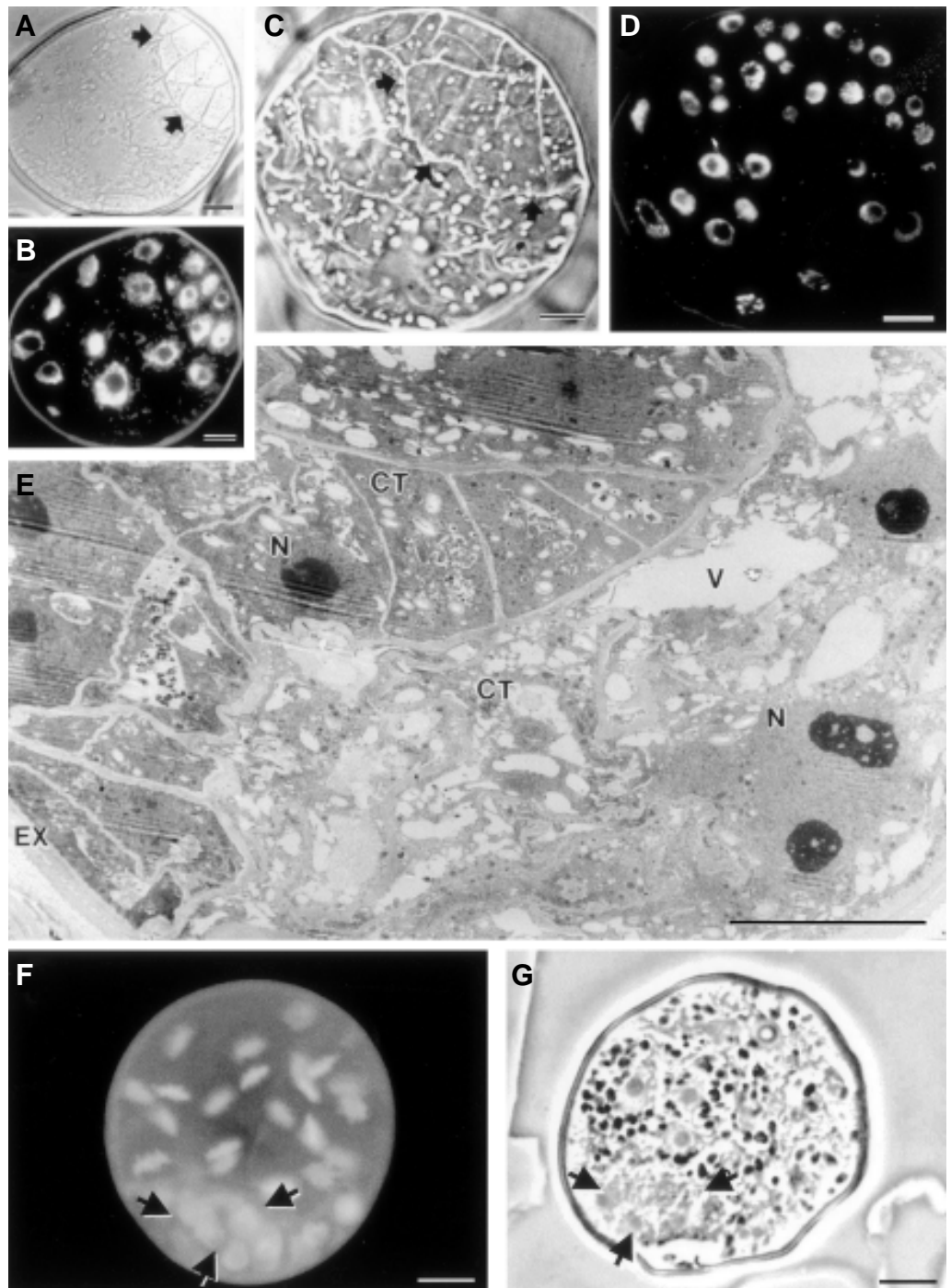
formed by numerous cells (Fig. 1G), and rounded structures formed by clusters of cells (Fig. 1F).

At any specific period of the culture some structures of the previous developmental stages were observed coexisting with the proembryo morphology typical of this stage, which once again revealed the asynchrony of the culture. For example, at 12 days, microspores, and multicellular proembryos surrounded by the pollen wall could be found (data not shown).

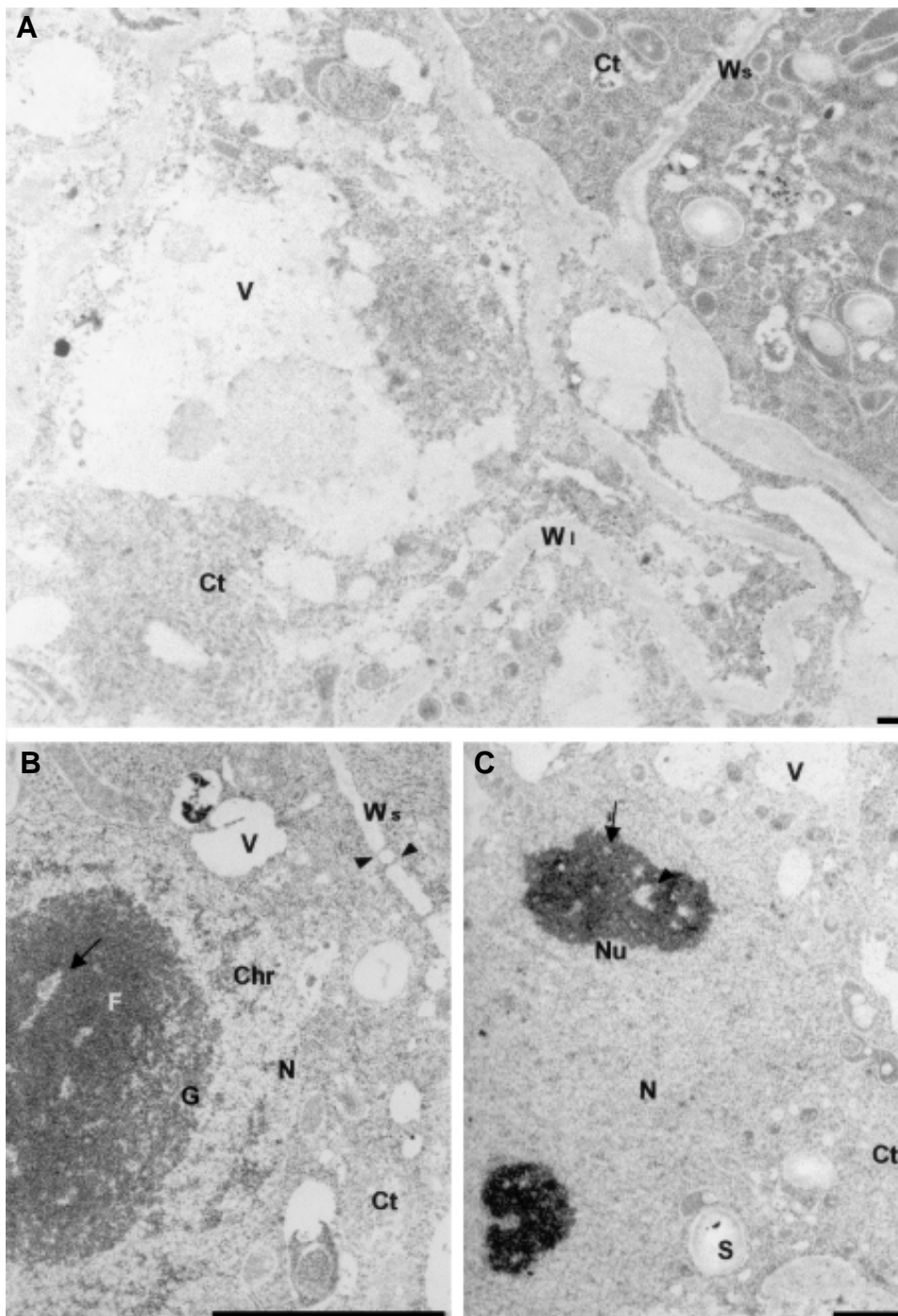
#### Ultrastructure of the Most Abundant 5/7 Day-Old Proembryos

The former observation of two different domains in the major part of 5/7 day-old proembryos in toluidine blue stained sections of conventionally processed samples, was confirmed on samples cryoprocessed in different ways. 1  $\mu$ m cryosections and Lowicryl semithin sections observed under phase contrast showed two domains: one smaller with many small cells separated by straight cell walls, and the other larger with wavy and incomplete cell walls and a highly vacuolate cytoplasm (Fig. 2A,B,C,D). DAPI staining of the same sections revealed that nuclei of the small domain were of a smaller size than those in the larger domain. In this domain nuclei displayed large nucleoli (Fig. 2 B,D). The proembryos showing this morphology had a similar size, between 75 and 85  $\mu$ m diameter. In general, the larger ones exhibited an increase in the size of the so-called small domain (Fig. 2 C,D).

At the electron microscopy (EM) level, noticeable differences in the ultrastructural cell organization were found between cells of the two domains. Cells of the small domain displayed dense cytoplasm with abundant ribosomes, endoplasmic reticulum, plastids, mitochondria and other organelles, only very small and scarce vacuoles were present in them (Figs. 2E and 3). They usually contained one nucleus which occupied a high proportion of the cellular volume and was located in the middle of the cell. The functional organization of the nucleus was similar to that in proliferating cells, i.e. the pattern of



**Fig. 2. Structure of two-domain morphology of 5-day old microspore proembryos.** A, B and C, D: Pairs of DAPI and phase contrast images of the same proembryos, in cryosection (A, B) and Lowicryl section (C, D). Regions indicated by arrows show smaller nuclei and cells; this domain is larger in the proembryo in C, D. (E) Electron micrograph at low magnification showing the general ultrastructure of the two domains. The darker cells correspond to the small domain indicated by arrows in light micrographs. CT, cytoplasm; N, nucleus; V, vacuole; EX, exine, pollen wall. (F) Synchronic mitosis observed in the large domain. Numerous mitotic images are visible after DAPI staining in the large domain, whereas only interphasic nuclei are found in the small domain (arrows). (G) Cytochemistry for starch. Numerous cytoplasmic inclusions in the large domain are positively stained, revealing their starch content. No starch accumulation appears in the small domain (arrows). Bars in all micrographs represent 10  $\mu$ m.

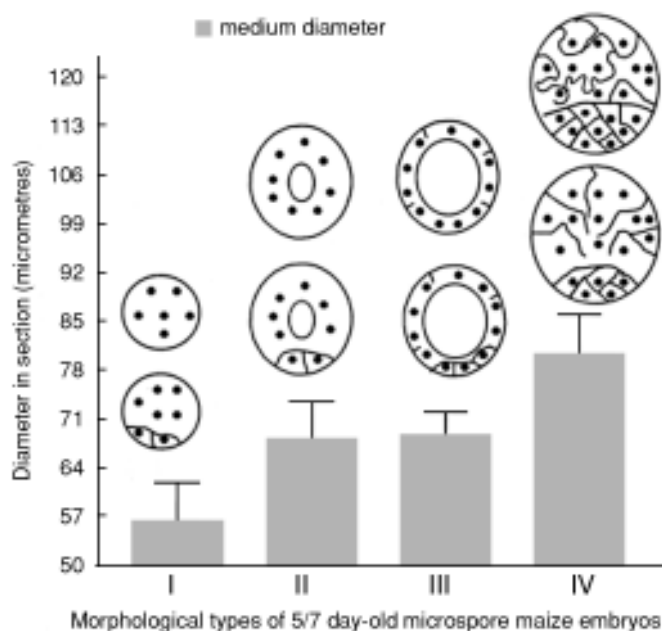


**Fig. 3. Differential ultrastructural features in the two domains of 5/7 day-old microspore maize proembryos.** (A) Cytoplasmic organization of cells of the two domains. Small domain cells (asterisks) exhibit dense cytoplasm with abundant ribosomes, plastids and other organelles; no large vacuoles are seen. Large domain cells show a clearer cytoplasm with numerous large vacuoles (V). Thicker and wavy cell walls ( $W_i$ ) appear in the large domain, in comparison with the thinner and straight walls ( $W_s$ ) of the small domain. (B, C) Nuclear organization of cells of the small (B) and large (C) domains. (B) Nuclei of the small domain (B) show small patches of condensed chromatin (Chr) and a large interchromatin region; some cells exhibit a large nucleolus with a granular component (G) surrounding the dense fibrillar one (F) and fibrillar centres (thin arrows). Plasmodesmata (arrowheads) are seen in the cell walls of this domain. (C) Nuclei of the large domain are larger, displaying very decondensed chromatin and a dense interchromatin region. The nucleoli show abundant granular component (G), homogeneous fibrillar centers (thin arrows) and central vacuoles of activity (arrowhead). S, starch. Bars represent 2.5  $\mu$ m.

chromatin was not very condensed with small patches and a large interchromatin region, the nucleoli exhibited various architectures, probably depending on the cell cycle period, some cells showed compact and small nucleoli, others presented granular component and fibrillar centres (Fig. 3B). Walls separating cells in the small domain were straight and of different thickness (Figs. 2E and 3A), showing numerous plasmodesmata (Fig. 3B).

The ultrastructural organization in the large domain was quite different. Cytoplasm showed numerous and large vacuoles, ribosome density was lower, providing it with a clearer electron density (Figs. 2E and 3A). One or various nuclei were observed in the same cytoplasm, wavy and, in some cases, incomplete walls were separating large cytoplasmic regions in this domain. Plastids were larger and many of them showed rounded and clear inclusions, probably starch granules. Specific cytochemistry for starch revealed large and numerous amyloplasts in the larger domain whereas none or very few were detected in the small domain (Fig. 2G). Cytoplasm also contained mitochondria, endoplasmic reticulum and other structures which were dispersed throughout this proembryo region (Figs. 2E and 3A). Nuclei were bigger and frequently displayed an ultrastructural organisation typical of high nuclear activity, like very decondensed chromatin, with a dense interchromatin region and nucleoli with an abundant granular component, homogeneous fibrillar centers and central vacuoles of activity (Fig. 3C).

In semithin sections, there were occasionally observed mitotic images in some proembryos. Mitosis in the large domain usually appeared in all their nuclei at the same time. This feature was observed in different morphological types, always in the coenocytic domain (Figs. 1E and 2F). In these proembryos, numerous mitotic chromosomes appeared in the large domain, whereas nuclei of the small domain were in interphase at the same time (Fig. 2F). When occasionally a mitosis was observed in the small domain, the rest of the nuclei were in interphase (data not shown).



**Fig. 4. Scheme representing the different morphological types of 5/7 day-old microspore maize proembryos.** Histogram showing the mean diameter of sections found in the four different categories of structures: morphological types I, II, III and IV, which were grouped by their morphological features summarized in the drawings and explained in detail in the text.

#### Other Morphological Types of 5/7 Day-Old Proembryos

Microscopical analysis of fractions enriched with 5/7 day-old microspore proembryos revealed the presence of various morphological types together with the main morphology described above. Regarding their size and main features, they were grouped into four different morphological types whose cellular organization and ultrastructure are described in the following paragraphs. Quantitative analysis of their size in section and a draft with their main characteristics are summarized in Fig. 4.

#### Morphological Type I

The smaller proembryos, 50–60 µm diameter, exhibited a coenocytic organization with several nuclei in a unique common cytoplasm where no cell walls were observed (Fig. 5 A,B,C). The cytoplasm contained many small organelles, numerous amyloplasts, vesicles and vacuoles which occupied the main cytoplasmic volume. In some sections whose cutting plane crossed a particular proembryo region, a different small region or domain could be observed in the Type I proembryos (Fig. 5C, arrows). It was situated

at the periphery and separated by a thick cell wall from the coenocytic domain; it contained only a few small uninucleate cells.

#### Morphological Types II and III

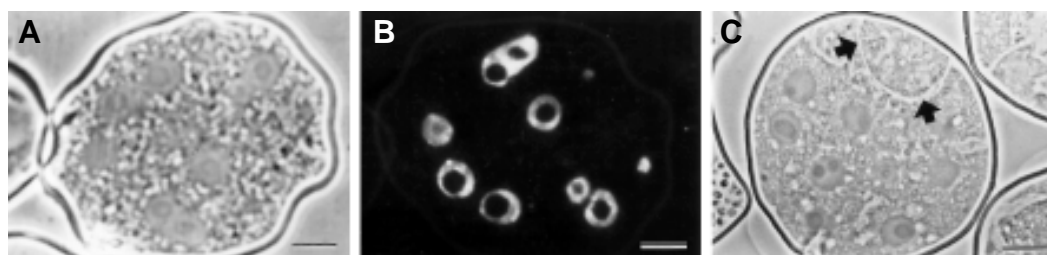
Another group of multicellular structures of 5/7 day-old proembryos were characterized by the coenocytic organization but showing a specific feature: the presence of a central clear region with either various large cytoplasmic vacuoles or a unique vacuole (Fig. 6 A,B,C,D). Their mean diameter was around 70 µm. In some of them (type II in Fig. 4) this central region did not occupy more than one third of the proembryo diameter, whereas other structures showed a much bigger central vacuole (type III in Fig. 4). The cellular organization around this central vacuole showed features similar to the coenocytic domain in morphological type I. In the structures with the biggest vacuoles some sinuous cell walls were found in a more or less radial direction, from the pollen wall to the central vacuole (Fig. 6 A,C). These walls showed uneven thickness. They showed free ends and appeared of heterogeneous organization, with a more dense inner region and a clear area close to the plasmalemma (inset in Fig. 6D). Numerous small vesicles, organelles and endomembranes appeared in the common cytoplasm, the nuclei displaying low chromatin condensation and nucleoli with a typical ultrastructural organization of high activity (Fig. 6D), as in the coenocytic domain in other proembryo morphological types. Similarly as in the previous morphological type, some sections also showed a small domain at the periphery, displaying a lens shape and limited by a thick cell wall (Fig. 6C, arrows). A few cells of similar morphology to those found in the small domain of other proembryo types appeared in this region (Fig. 6C).

#### Morphological Type IV

In this morphological type IV, the most abundant structures, previously described (Figs. 2 and 3) were classified. The structures of this type exhibited the higher sizes in the culture, from 75 to 85 µm mean diameter. They were characterized by the absence of large central vacuoles, a small domain bigger than in the morphological types I to III, and the presence of abundant and incomplete wavy walls in the large domain (Figs. 2 and 3).

#### Cytochemical and Immunocytochemical Analyses of Specific Cellular Components 5/7 Day-Old Proembryos

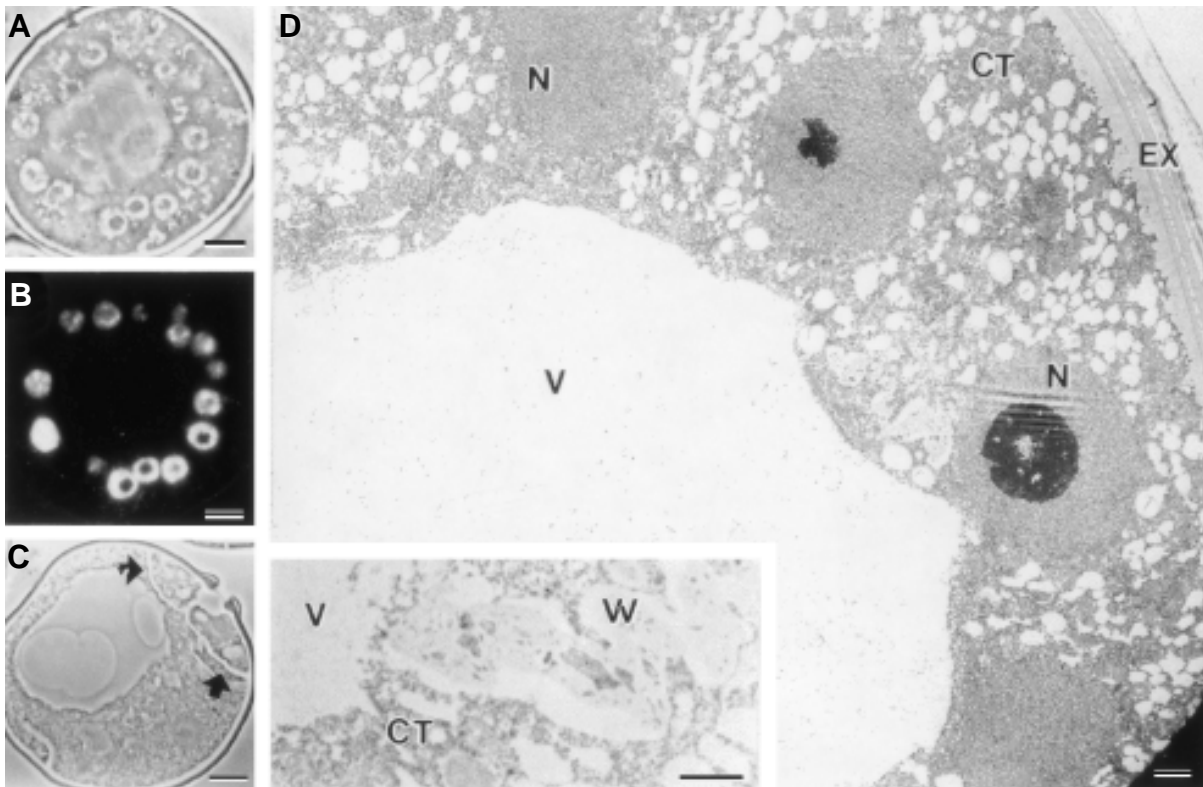
The presence of two regions or domains with specific structural characteristics were found in all morphological types of proembryos, even though the reduced size of the so-called small domain, specially in morphological types I to III, made its observation in sections difficult, and a question of chance depending of the cutting plane. To further characterize these two



**Fig. 5. Structure of microspore proembryos of morphological type I.** (A,B) A semithin section of the same proembryo observed under phase contrast (A) and DAPI (B) staining, Coenocytic organization. This

section showed no small domain. (C) A semithin section under phase contrast. A small region separated by a thin wall (arrows) is observed. The rest of the structure exhibited a coenocytic organization similar to that in A,B. Bars represent 10 µm.





**Fig. 6. Structure of microspore proembryos of morphological type III.** (A,B) A semithin section of the same proembryo observed under phase contrast (A) and DAPI staining (B). A large vacuole occupies the centre of the proembryo, nuclei are ordered in line around it, immersed in a peripheral layer of cytoplasm; in this section no small domain is observed. A few thick walls appear in contact with the exine following a radial but irregular direction. (C) Semithin section under phase contrast. A lens-shaped small region separated by a thin wall (arrows) is observed at the periphery, the rest of the structure is similar to that in A,B, with a big central vacuole and a few thick radial walls. (D) Ultrastructure of proembryos of morphological type III. The cytoplasm (CT) contains numerous vesicles, small vacuoles and organelles. Nuclei show a structural organization similar to that found in the large domain in any morphological type, with decondensed chromatin and a big nucleolus. The inset shows at higher magnification a cytoplasmic (CT) region containing a radial thick wall (W) with a heterogeneous structure. V, vacuole; EX, exine. Bars: A,B,C, 10  $\mu\text{m}$ ; D, 1  $\mu\text{m}$ .

domains, several cytochemical and immunocytochemical assays were done, results revealing new differences between both proembryo regions.

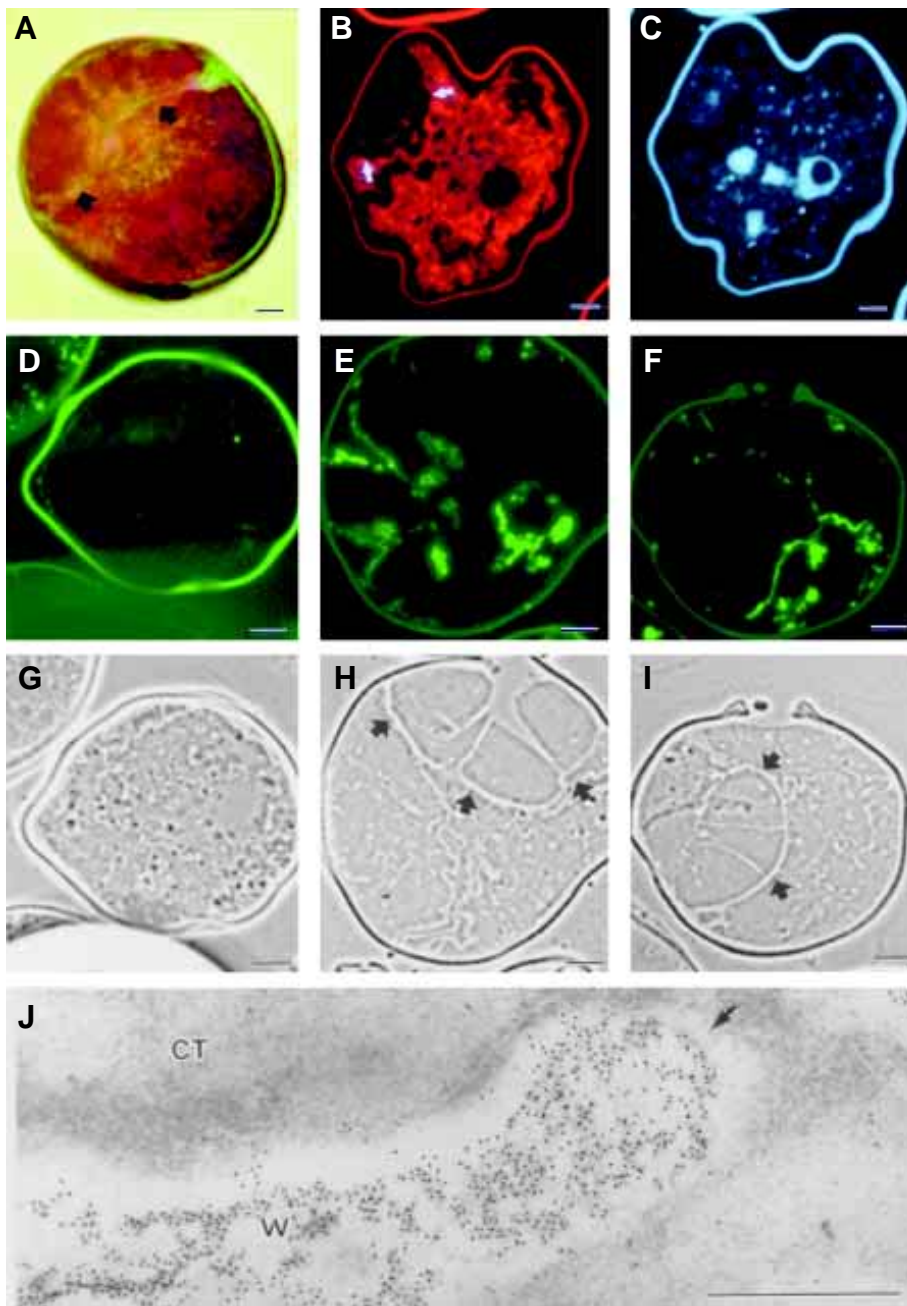
**Cytoskeleton Components:** the presence of tubulin, as a marker of cell divisions and evidence of growth and morphogenesis (Brown *et al.*, 1994) in the developing proembryos, was studied by using anti- $\beta$ -tubulin antibodies on semithin cryosections for immunofluorescence assays, DAPI staining was also applied to reveal nuclei in these preparations. An homogeneous and intense immunofluorescence was observed in the cytoplasm of the large domain, the nuclei, wavy cell walls and small cytoplasmic vacuoles and organelles appeared completely negative (Fig. 7 B,C). The small domain also showed a positive anti-tubulin signal on the cytoplasm of their cells but the intensity of the fluorescence was lower than that in the large domain (Fig. 7 B,C), which would indicate differences in the presence of tubuline in both proembryo regions. The peripheral microspore wall, the exine, exhibited unespecific autofluorescence with all types of irradiation, appearing visible in DAPI and immunofluorescence images (Fig. 7 B,C,D,E,F).

**Specific Cell Wall Components:** since cell wall structure was different between both domains and occasional wavy and incomplete walls appeared in one of them, special attention was devoted to

the study of these structures. Anti- $\beta$ -1,3-glucan antibodies recognizing this polysaccharide, the callose, was used for immunofluorescence and immunogold labelling. Specific immunofluorescence was observed in certain walls of the large domain, although no signal appeared in the small domain (Fig. 7 E,H,F,I). In proembryos of the morphological type I, with no cell walls in the coenocytic domain, anti-callose immunofluorescence was completely negative (Fig. 7 D,G). Anti-callose immunogold labelling did not label the straight walls of the small domain (data not shown), but provided specific signals to certain regions of the wavy walls (Fig. 7J). Gold particles were decorating the inner part of certain regions of these walls, leaving some others absent of labelling. Labelling was especially high in thick regions of the wavy walls, and frequently near a free end (Fig. 7J).

#### **In Situ Expression of Embryogenesis-Specific Genes**

The expression of *ZmAE1* and *ZmAE3* embryogenesis-specific genes (Magnard *et al.*, 2000) has been analyzed in the different morphologies and cell types characterized in 5/7 day-old microspore proembryos. Non-isotopic *in situ* hybridization (ISH) was performed at both light and electron microscopy levels. Semithin Lowicryl sections were used for ISH since they helped to clearly distinguished the various cell types and domains. The accessibility of nucleic acids to probes for ISH at light microscopy is limited. To visualize the



**Fig. 7. Anti-tubulin (B,C) and anti-callose (D-J) immunolocalizations in the two-domain structures.** (A) Proembryo exhibiting a small domain (arrows) and a large domain, clearly distinguished. Alexander staining in squash. (B,C) Anti- $\beta$ -tubulin immunofluorescence (B) and DAPI staining (C) of the same structure on a semithin cryosection. Positive immunofluorescence is observed in the cytoplasm, some dark areas appear corresponding to nuclei, walls and small organelles. A more intense fluorescence signal is observed in the cytoplasm of the large domain than in the small one (arrows). D-I: Pairs of anti-callose and phase contrast images (situated in columns) of the same proembryos. (D,G) Morphological type I, coenocytic organization; no signal was observed with anti-callose antibodies. (E,I) Morphological type IV, two domains and wavy walls in formation in the large domain. The small domain (arrows in phase contrast images) does not exhibit immunofluorescence. Some walls of different thicknesses show a positive anti-callose signal in the large domain. (J) Anti-callose immunogold labelling. Numerous gold particles decorate some of the thick wavy walls of the large domain, many of them with free ends. Gold particles are forming a thick network mostly at the inner part of the wall. Bars: A-I, 10  $\mu$ m; H, 0.5  $\mu$ m.

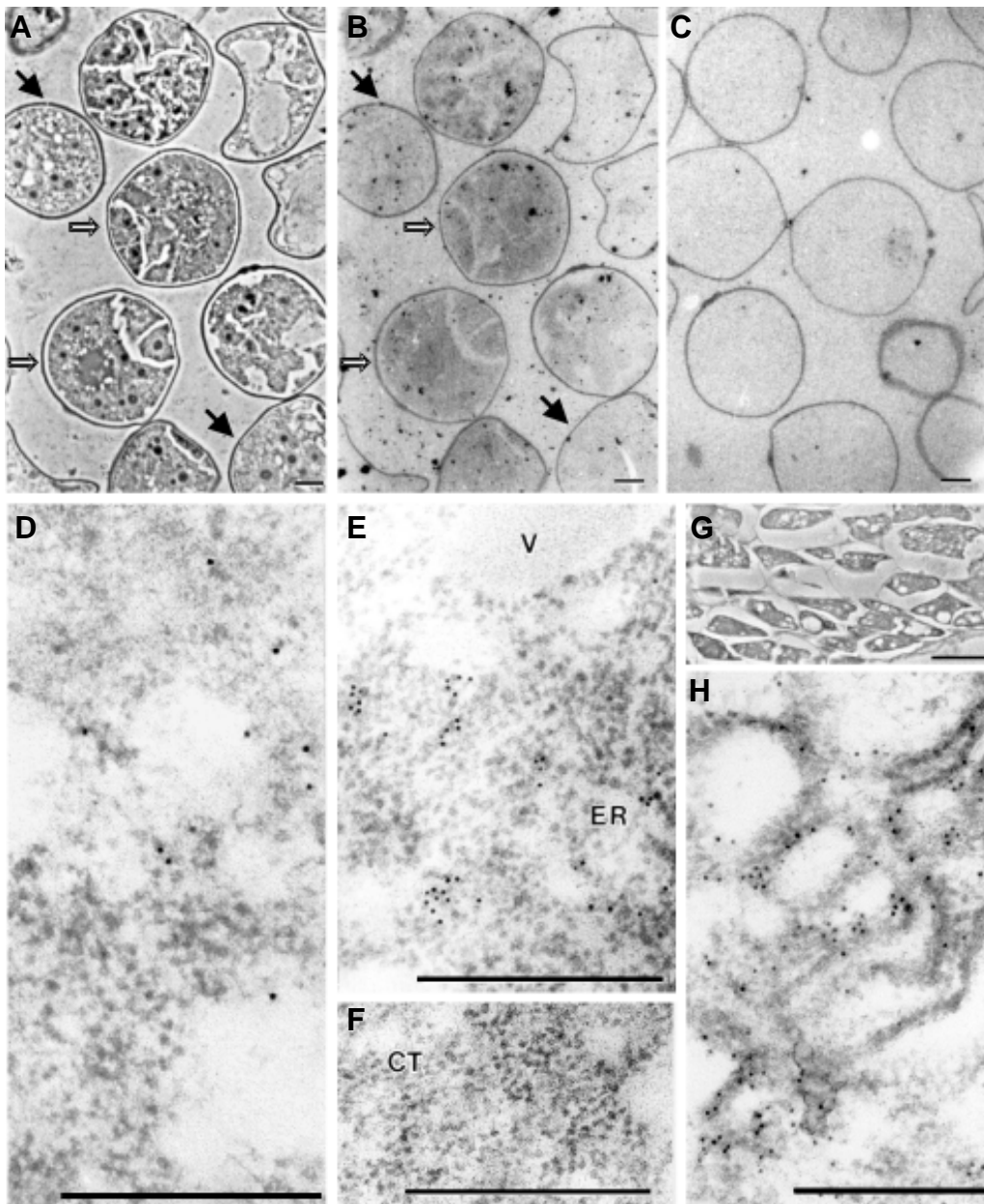
hybrids, immunogold labelling of the hybridized probes followed by silver enhancement was used. A similar pattern of ISH results were obtained with both genes *ZmAe1* and *ZmAe3*.

A positive hybridization signal in the two domains was obtained in all morphological types of 5/7 day-old proembryos (Fig. 8 A,B). Controls with sense probes never provided a signal in any proembryo (Fig. 8C). Proembryos displaying the organization of morphological type I, i.e. the coenocytic organization (black arrows in Fig. 8 A,B), exhibited a lower hybridization signal than other morphological types (white arrows in Fig. 8 A,B).

Ultrastructural ISH, developed by an immunogold technique, was performed as it has a higher sensitivity and enables the quantification of the signal. When it was applied to 5/7 day-old proembryos results showed the presence of *ZmAe1* and *ZmAe3* transcripts in ribosome-rich areas of the cytoplasm (Fig. 8 D,E), whereas organelles, vacuoles and cell walls appeared almost free of labelling. Lower labelling density was found in cells of type I proembryos (Fig. 8D) than in those of other morphological types, II, III and IV (Fig. 8E). Control experiments with sense probes did not show significant labelling (Fig. 8F). Ultrastructural ISH was also performed on zygotic endosperm cells of maize caryopses 7 days after pollination (7DAP), specifically in the embryo surrounding region, Esr (Fig. 8G), in which expression of both embryogenesis-specific genes has been reported (Magnard *et al.*, 2000). Ultrastructural ISH results showed numerous gold particles decorating ribosome-rich areas of the cytoplasm, frequently surrounding the abundant vesicles and wide ER cisterns typical of these Esr cells (Fig. 8H).

#### Quantitative Study of Ultrastructural *In Situ* Hybridization

The labelling density was estimated in different cell types as the number of gold particles per area unit ( $\mu\text{m}^2$ ), obtaining very similar results for both genes. Proembryo types were grouped into two categories for the quantification: type I proembryos and type II, III and IV proembryos. The results of the quantitative evaluation of ISH experiments are shown in Fig. 9. Very low levels of background (averages of 3.02-6.82 particles/ $\mu\text{m}^2$ ) were obtained for the three cell types. The labelling density mean value in microspore proembryos type I, coenocytic organization, was much lower ( $18.17 \pm 8.70$  particles/ $\mu\text{m}^2$ ) than this value in other morphological types ( $139.83 \pm 51.38$  particles/ $\mu\text{m}^2$ ). On the other hand, ISH labelling density in Esr cells was high, the mean value being  $146.08 \pm 42.20$



**Fig. 8.** *In situ* hybridization (ISH) of **ZmAE1** and **ZmAE3** embryogenesis-specific genes. (A-C) ISH of **ZmAE1** transcripts over semithin sections developed by immunogold followed by silver enhancement. A and B are the same section observed under phase contrast, (A) to visualize the structure, and bright field (B) to visualize the silver precipitates. Hybridization signal is observed in cells of the two domains. Hybridization signal is slightly lower in type I proembryos with coenocytic organization (black arrows) than in type II-IV proembryos (white arrows). In control experiments, ISH with the sense probe (C) does not provide any labelling. (D-G) Ultrastructural ISH of **ZmAE3** (D-F) and **ZmAE1** (G) transcripts on ultrathin sections of microspore proembryos type I (D) and type IV (E) and zygotic endosperm Esr cells of 7DAP caryopses (G,H). Hybridization signal appears as abundant gold particles, frequently in linear arrays, over cytoplasmic regions (CT); no labelling was observed in the nucleus, vacuoles (V), endoplasmic reticulum cisterns (ER) or other organelles. No labelling was observed in controls with sense probes (F). Bars: A,B,C,G, 10  $\mu\text{m}$ ; D,E,H, 0.5  $\mu\text{m}$ .

particles/ $\mu\text{m}^2$ . Statistical tests indicated that ISH labelling density in microspore proembryos type I were significant in comparison with background values (student's T test,  $p \leq 0,01$ ). Moreover, no significant differences (T test,  $p \leq 0,01$ ) were probed between ISH results in microspore proembryo types II, III and IV, and zygotic endosperm Esr cells.

Control experiments with sense probes provided very low labelling density mean values (average 1.95 particles/ $\mu\text{m}^2$ ).

## Discussion

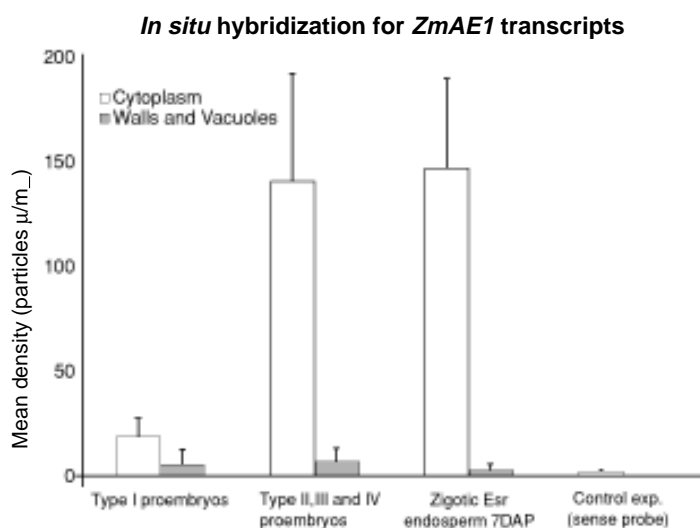
### **Presence of Two Different Domains in Young Microspore Proembryos**

In this work, various cytochemical and immunocytochemical methods have been carried out to characterize the structural organization

of young microspore maize embryos, the results providing new insights into the initials of the process and its parallelism with zygotic embryogenesis. The results obtained built, for the first time, a detailed cellular and ultrastructural characterization of the young microspore proembryos in maize.

An interesting feature, which is differential from dicot systems, has arisen from the results presented here: the existence of two domains with very different sizes and cellular characteristics, in the young maize proembryos. The presence of different cell types in the first steps of pollen embryogenesis has been described in other cereals in a few reports (Sunderland *et al.*, 1979; Sunderland and Huang, 1985, Huang, 1986). Only in maize, recently our previous preliminary papers have indicated the presence of two different cell types (Domenech *et al.*, 1998; Góralski *et al.*, 1999; Magnard *et al.*, 2000, Matthys-Rochon 2002). Raghavan, (1975) first reported two regions





**Fig. 9. Quantification of the high resolution *in situ* hybridization signal of *ZmAE1* transcripts.** Histogram showing the mean labelling density in ordinates as the number of gold particles per square micrometer in the cytoplasm (white bars) and in vacuoles and cell walls (gray bars), as an estimation of the background level. Signal was quantified in two categories of structures: Type I embryos, which showed a low number of gold particles, and Type II, III and IV embryos, all of which exhibited high labelling density. The third column corresponds to signals in the zygotic embryo-surrounding region of the endosperm, 7 days after pollination. Labelling obtained in control experiments (control exp.) using sense probes was very low.

showing different cellular characteristics in young microspore embryos of *Hyoscyamus niger*, one of them was suggested to be homologous to the suspensor in zygotic embryogenesis (Raghavan, 1975; Raghavan, 1986). In barley, multicellular structures with two regions showing different staining properties, at light microscopy levels, were found and putative homologies between these two regions and the embryo and endosperm or suspensor were hypothesized (Sunderland *et al.*, 1979). Some ultrastructural observations also revealed a few differences between cells of the two regions, in barley (Sunderland and Huang, 1985; Huang, 1986), which will be discussed later. Also in barley, a recent paper has reported the existence of two different cell types on early microspore-derived embryos (Ramírez *et al.*, 2001a). Some of these reports suggested different possibilities for the origin of both regions from microspores, vegetative and generative cells, indicating almost all origins to be possible (Sunderland and Huang, 1985; Huang, 1986). The origin of the large and small domains in maize microspore embryos cannot be indicated in this study, further work will be necessary to answer this question.

The results of the ultrastructural, cytochemical and immunocytochemical assays revealed striking differences between the cellular organization of the two domains. Moreover, results seem to indicate that the so-called large domain is sharing characteristics with the endosperm in zygotic embryogenesis. Our previous report (Magnard *et al.*, 2000) also reported similarities to endosperm-like functions in early microspore maize embryos. In the following paragraphs, it will be named: "endosperm-like domain".

Zygotic endosperm development in maize, as well as in most cereals, is characterized by a first period in which nuclear divisions without cytokinesis occur and give place to a polynucleate, coeno-

cytic, structure which, in the initial stage, exhibits a large central vacuolate area, the nuclei being situated at the periphery with a layer of cytoplasm (Mares *et al.*, 1975; Mares *et al.*, 1977; Van Lammeren, 1986b; Olsen *et al.*, 1995; Olsen, 1998). In subsequent developmental stages, the vacuolate region disappears and the zygotic endosperm starts to form cell walls in a peculiar form, with no standard cell plate, and the so-called "free-growing" walls appear. They begin their formation at the periphery and begin randomly ingrowing, producing irregular cytoplasmic pockets which contain different numbers of nuclei until the completion of the so-called "cellularization" process of the endosperm (Mares *et al.*, 1975; Morrison and O'Brien, 1976; Mares *et al.*, 1977; Vijayaraghavan and Prabhakar, 1984; Brown *et al.*, 1994; Olsen *et al.*, 1995). The cellular organization of the endosperm-like domain in microspore proembryos appears very similar to the endosperm structure, at various developmental stages: a whole coenocytic structure and no walls, large central vacuole and peripheral nuclei, and the presence of cell walls with free ends (Mares *et al.*, 1975; Morrison and O'Brien, 1976; Mares *et al.*, 1977; Van Lammeren, 1986b; Brown *et al.*, 1994; Olsen *et al.*, 1995). In multicellular pollen grains appearing in the embryogenesis anther culture of barley and wheat, the presence of wall fragments and incomplete walls attached to the intine have been reported, together with normal cell plate formation (Sunderland and Huang, 1985; Huang, 1986). The presence of numerous polysaccharidic inclusions, cytochemically identified by starch granules, in the cytoplasm of the large domain also draw endosperm tissue near to this region which is highly active in producing this storage product, being full of starch granules for later developmental stages (Vijayaraghavan and Prabhakar, 1984).

As  $\beta$ -1,3-glucan, callose, is an unusual polysaccharide which is specially abundant in the so-called "growing-cell walls" of the endosperm (Morrison and O'Brien, 1976; Brown *et al.*, 1994), immunocytochemical assays with anti-callose antibodies were performed. Results revealed the presence of this unusual polysaccharide in the walls of the endosperm-like domain, but not in the other domain.

Occurrence of synchronous mitosis has been reported during endosperm development initials (Van Lammeren, 1986b; Olsen, 1998). This phenomenon, had previously been observed in microspore multicellular proembryos of maize by us (Domenech *et al.*, 1998; Magnard *et al.*, 2000), and also supports the homology between the large domain and the zygotic endosperm.

Differences in tubulin immunofluorescence signals were also observed between the two domains, which could indicate a different amount of tubulin between the two regions. This finding could indicate differences in the dynamics and organization of the microtubular network, maybe related to the free-ends of the growing cell walls, only present in the large domain, as already suggested for the zygotic endosperm cells (Olsen *et al.*, 1995). Our results detecting more presence of tubulin in the endosperm-like domain of the microspore proembryos could be related to an additional function of microtubules in guiding flotant cell wall formation, as reported by Brown *et al.*, (1994) during early zygotic endosperm development as well as in the orientation in different planes of the numerous divisions occurring at that time (Russell, 1993; Brown *et al.*, 1994, 1996, Olsen, 1998). These defined features are only present in the zygotic endosperm, but not in the embryo proper; their presence in the large domain of the microspore proembryos supports once more the homology between the large domain and the zygotic endosperm.

The structural organisation of nuclei in the large domain involved a decondensed chromatin pattern and nucleoli with an abundant granular component and fibrillar centers; this structural organisation reflects high nuclear activity in transcription and ribosome biogenesis (Risueño and Medina, 1986; Risueño and Testillano, 1994), which would be consistent with the endosperm-like domain as an active tissue in biosynthesis of different components for cell walls and storage products.

The small domain, contrary to the endosperm-like characteristics observed in the large domain, has shown cellular features clearly characteristic of proliferating cells: more dense cytoplasm with scarce vacuoles, small and polygonal cells with a large nucleus which can be found exhibiting a different structural organisation, probably depending on their cell cycle phase (Risueño and Medina, 1986), straight and not very thick walls with plasmodesmata, etc. These characteristics are homologous to cells of the embryo proper in the first stages of zygotic embryogenesis in which zygote undergoes several divisions with normal cytokinesis and cell plate formation (Natesh and Rau, 1984; Van Lammeren, 1986b; Van Lammeren, 1986a). In microspore-derived embryogenesis through anther cultures of barley and wheat, the dense region observed in multicellular pollen grains has been reported to show ultrastructural meristematic characteristics (Sunderland and Huang, 1985; Huang, 1986).

#### **Early Microspore Embryogenesis parallels Zygotic Embryogenesis in the Initial Stages**

In zygotic embryogenesis, the beginning of development and nuclear mitosis in endosperm is prior to divisions taking place in the zygote (Natesh and Rau, 1984; Van Lammeren, 1986b; Van Lammeren, 1986a). The fact that only a few cells were observed in the small domain in most proembryos, whereas in the largest proembryos an important increase in the volume occupied by the embryo-like domain was observed, would be in agreement with a parallel between initial endosperm development and morphological types I-III, and establish a new structural homology between microspore and zygotic embryogenesis in maize. Taking into account, on one hand the known asynchrony in microspore embryogenesis cultures, which have clearly been observed in the culture system used in this work (Gaillard *et al.*, 1991; Magnard *et al.*, 2000) and on the other hand the homologies between structural features of endosperm-like domain and zygotic initial endosperm development, it could be hypothesised that the four morphological types described here represent different stages in microspore-derived embryo development. Studies currently in progress, with tracking experiments will shed light on this question.

The data reported here show many common cellular features between both processes, some of them are not frequent and quite characteristic of endosperm development; therefore, it will not be easy to explain this set of similarities excluding any functional parallelism between early microspore embryogenesis and endosperm and embryo development. The use of molecular markers, specific for endosperm or embryo during early development would be of special interest to analyse their expression during microspore embryogenesis but in cereals, few markers have been identified, principally in barley (Doan *et al.*, 1996; Mena *et al.*, 1998; Vicente-Carbajosa *et al.*, 1998; Oñate *et al.*, 1999). Only a few genes have been reported to be specifically expressed

during very early zygotic embryogenesis in maize (Opsahl-Ferstad *et al.*, 1997; Magnard *et al.*, 2000). *ZmAE1* and *ZmAE3* have recently been isolated, they were reported to be expressed during early development of both microspore and zygotic embryogenesis but they did not show any expression during pollen development or in other vegetative tissues (Magnard *et al.*, 2000). *In situ* hybridisation experiments resulted in the expression of these two genes in specific regions of the endosperm, the so-called *Embryo Surrounding Region* (Esr) (Opsahl-Ferstad *et al.*, 1997), at very early developmental stages, from 3 to 12 days after pollination (Magnard *et al.*, 2000). In 5/7 day-old proembryos expression of *ZmAE1* and *ZmAE3* in the two domains has also been reported (Magnard *et al.*, 2000). Our results revealed expression of both genes in the four morphological types described for 5/7 day-old proembryos; this result further supported that those different structures found in the culture were following the embryogenesis developmental program and were not representing aberrant or rare developmental pathways.

On the other hand, the quantification of the *in situ* hybridisation (ISH) signals permit the comparison of the expression levels among the different morphological types of microspore proembryos and the Esr of zygotic endosperm 7 days after pollination, which is the developmental period with a known high expression of both genes *ZmAE1* and *ZmAE3* (Magnard *et al.*, 2000). The quantitative data shows a similar expression level in Esr cells and microspore embryos of type II, III and IV. A much lower expression was detected in the coenocytic embryos of type I. If the morphological type I embryos represent a earlier developmental stage for the microspore embryogenesis, the lower level of expression at this period suggests, once more, a parallelism with the coenocytic endosperm of the early zygotic embryogenesis, in which no expression of both and/or other related genes has been reported (Opsahl-Ferstad *et al.*, 1997; Magnard *et al.*, 2000). This fact is also in agreement with the hypothesis of the existence of an endosperm-like function during early microspore embryogenesis.

## **Materials and Methods**

### **Plant Material**

The material used were five and seven day-old microspore proembryos of *Zea mays L.*, genotype DH5 x DH7. These proembryos were obtained from microspores *in vitro* cultures. The conditions for the isolation of microspores, *in vitro* cultures and embryogenesis induction were those previously reported by (Gaillard *et al.*, 1991). Fractions enriched in 5/7 day-old proembryos were obtained by filtration and centrifugation in Percoll gradient as described by (Magnard *et al.*, 2000).

### **Conventional Processing for Electron Microscopy**

Samples were fixed in Karnovsky (5% glutaraldehyde and 4% formaldehyde) in 0.025 M cacodylate buffer for 5 h, embedded in 15% gelatine and cut into small squares (1mm x 1mm). Samples were dehydrated in an ethanol series and embedded in Epon. Ultrathin sections were mounted on Formvar-coated copper grids and stained first with uranyl acetate for 30 min and after with lead citrate for 2 min and observed in a JEOL 1010 EM at 80 kV.

### **Lowicryl Embedding**

Samples were fixed in 4% formaldehyde in phosphate buffered saline (PBS), pH 7.3 at 4°C overnight. After washing in PBS, they were

dehydrated in methanol series by Progressive Lowering of Temperature (PLT). Some of the samples were subjected to the methylation-acetylation (MA) method (Testillano *et al.*, 1995b). Finally all the samples were infiltrated and embedded in Lowicryl K4M resin at -30°C and polymerized under UV irradiation. Lowicryl ultrathin sections were mounted on formvar-coated nickel grids and used for immunogold labelling and *in situ* hybridization.

#### **Cryofixation and Cryoultramicrotomy**

Samples were cryofixed and cryosectioned for immunofluorescence purposes as previously described (Testillano *et al.*, 1995a). Samples were cryoprotected with 2.3 M sucrose and cryofixed by plunging into liquid propane at -160°C. They were stored in liquid nitrogen until cryoultramicrotomy; then they were cut at -80°C obtaining 1 mm cryosections which were placed in a sucrose drop over aminopropyl-triethoxysilane (Sigma, St. Louis, MO/USA) coated slides, and stored at -20°C until used for immunofluorescence assays

#### **Starch Cytochemistry**

A staining solution containing 2% potassium iodide and 0.2% iodine in water (O'Brien and McCully, 1981) was applied for 2-5 minutes to semithin Lowicryl sections. After rinsing in water and air-drying, sections were mounted on Eukitt and observed under bright field.

#### **Probes and Antibodies**

For *in situ* hybridization, digoxigenin-labelled RNA probes were synthesized by *in vitro* transcription of the corresponding *ZmAE1* and *ZmAE3* cDNA clones (Magnard *et al.*, 2000). Sense riboprobes were also synthesized to be used as controls.

For immunocytochemistry, the antibodies used were: anti- $\beta$ -1,3-glucan, mouse monoclonal IgG (Biosupplies, Parkville Victoria, Australia); anti-tubulin  $\alpha$  Ab-2, clone DM1A, mouse monoclonal antibody (Neomarkers, Union City, California/USA).

#### **Immunofluorescence**

The procedure used was as previously described (Testillano *et al.*, 1995a). Slides carrying the cryosections were immersed in PBS to remove the sucrose. After blocking with 10% fetal calf serum (FCS) in PBS for 5 minutes they were incubated with the first antibody for 1 h diluted 1:25 in 5% FCS in PBS for the anti- $\alpha$  tubulin; 1:10 for anti- $\beta$ -1,3-glucan. After washing in PBS, they were incubated with goat anti-mouse IgG antibody conjugated with either Cy2 or Cy3 fluorochromes (Molecular probes, Eugene, OR, USA) diluted 1:25 in 5% FCS in PBS for 45 min in darkness. After washing in PBS and in distilled water, DAPI staining was performed. The cryosections were mounted on Mowiol, and observed in a Zeiss Axioplan fluorescence microscope equipped with a CCD camera (Photometrics, Tucson, Arizona, USA). Images were recorded by CCD camera, digitalized and printed in a Epson Stylus Photo printer, on high quality glossy paper. Controls were made by replacing the first antibody with 5% FCS in PBS.

#### **Immunogold Labelling**

This experiment was performed essentially as previously described by (González-Melendi *et al.*, 1995). Grids carrying Lowicryl ultrathin sections from MA-samples were floated for 5 min in 5% Bovine Serum Albumine (BSA) in PBS. Then, they were incubated for 1 h at room temperature with anti- $\beta$ -1,3-glucan monoclonal antibody diluted 1:50. After three washes with PBS sections were incubated with goat anti-mouse IgG antibodies conjugated to 10 nm colloidal gold (BioCell, Cardiff, UK) diluted 1:25 in 1% BSA in PBS, for 45 minutes. Then, the grids were washed in PBS and double-distilled water and air dried. Finally, sections were counterstained with 5% aqueous uranyl acetate for 20 minutes and lead citrate for 10 seconds and observed in a JEOL 1010 electron microscope at 80 kV. Controls were made by not using the antibody during the first incubation.

#### **In Situ Hybridization**

At light microscopy level, *in situ* hybridization was performed on semithin Lowicryl sections using digoxigenin-labelled RNA probes. The sections were placed over aminopropyltriethoxy-silane coated slides. They were hydrated with distilled water for some seconds and pretreated with 1  $\mu$ g/ml proteinase K in 0.1 M Tris-HCl, pH 7.5 containing 50 mM EDTA, for 1h at room temperature. After three washes in the buffer, the sections were acetylated by treatments with 0.1 M triethanolamine for 5 min and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Then, they were washed in SSC and distilled water and were incubated with 25  $\mu$ l of hybridization solution, at 50°C for 48 h. To avoid evaporation, drops of hybridization solution were covered with a coverslip and sealed with silicon. Hybridization solution consisted of a digoxigenin-labelled RNA probe diluted 1:25 in the hybridization buffer (50% formamide, 10% dextran sulfate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 500 mM NaCl, 1% Denhard's solution, 200  $\mu$ g/ml yeast tRNA). The sections were washed twice in 2 x SSC for 5 min and twice in 0.2 x SSC for 15 min at 50°C. The visualization of the hybrids was performed by immunogold labelling with anti-digoxigenin-gold antibodies followed by silver enhancement to amplify the signal. The sections were then washed for some minutes in PBS and 5% BSA in PBS for 5 minutes. They were then incubated with goat antidigoxigenin conjugated with 10 nm colloidal gold (BioCell, Cardiff, UK) diluted 1/25 in 1% BSA in PBS for 45 min. The signal was amplified using a silver enhancement kit (BioCell, Cardiff, UK).

At electron microscopy level, the method used was described by (Préstamo *et al.*, 1999). Digoxigenin-labelled RNA probes were used diluted at 1:20 in hybridization buffer consisting of 50% formamide, 10% dextran sulfate, 10mM Tris, 1M EDTA, 500 mM NaCl and 200  $\mu$ g/ml yeast tRNA. Hybridization was performed overnight at 50°C, and hybrids were visualized by immunogold labelling with anti-digoxigenin antibodies conjugated with 10 nm colloidal gold.

Controls were made replacing the antisense RNA probe by a sense probe at the same concentration.

#### **Quantification of the Hybridization Signal**

To evaluate *in situ* hybridization signal, 15-25 micrographs were analyzed in each cell type. The micrographs were randomly obtained from various cells and grids. Cytoplasmic areas were randomly selected and outlined, and each area was estimated using a specific software (OPTIMAS, USA). The number of gold particles in each area was hand-counted and labelling density was measured as to the number of particles per square micrometer. The minimum sample size per each cell type was determined by the progressive mean technique (confidence limit 1%). The labelling density in vacuoles and cell walls was taken as the inner control of the background level. The same quantification was performed on micrographs from control experiments with sense probes. The statistical significance of labelling density differences among cell types was assessed using a Student's t test.

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