

## The early microspore embryogenesis pathway in barley is accompanied by concrete ultrastructural and expression changes

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**ABSTRACT** The use of dihaploid plants for obtaining new varieties has been widely reported in different plant species. The regeneration of these plants is carried out by *in vitro* induction of embryogenesis in microspores and pollen grains. This process is switched by the application of stress treatments and hormones, but the efficiency is still very low in many crops. The molecular and cellular processes responsible of the change in the developmental program of the microspore are still under investigation. Defined ultrastructural and expression changes have been reported to accompany the reprogramming of the microspore to embryogenesis in dicot systems (Testillano *et al.*, 2000), but less is known on the cellular characterization of the process in monocots.

In this work, microspore embryogenesis has been induced by *in vitro* cultures of *Hordeum vulgare* L. Cv Igri after osmotic treatment (Cistué *et al.*, 1994). To characterize the developmental pathway followed during microspore embryogenesis induction in barley, early stages of the process have been studied by a correlative microscopy approach at both light and electron microscopy levels. Samples at the beginning and after 1, 3, 6 and 9 days of culture were processed and embedded in Epon for ultrastructural analysis, and cryoprocessed and cryoembedded in Lowicryl K4M for immunogold labelling (Testillano *et al.*, 1995a)

Results showed that most of the cells which responded to the stress treatment were vacuolate microspores (Fig. 1a) indicating that this developmental stage is responsive for embryogenesis induction in barley. Vacuolate microspore has been also reported to be the most efficient developmental stage for embryogenesis induction in various dicot species (González-Melendi *et al.*, 1996). At this stage, a large cytoplasmic vacuole occupied a high proportion of the cell volume and the nucleus was located at the periphery (Fig. 1a). The nucleus displayed a highly decondensed chromatin pattern and one or two small nucleoli and the cytoplasm appeared clear (Fig. 1a). After the first embryogenic division, various cells containing large nuclei and scarce cytoplasm were found at peripheral locations, large vacuoles were still present at very early stages (Fig. 1b). Cytoplasm contained numerous ribosomes and the organelles and the nuclei exhibited a similar organization as in the vacuolate microspore. At later stages, subsequent divisions occurred and multicellular proembryos still surrounded by the microspore wall, the exine, were observed

(Fig. 1 c,d). Different cellular organizations appeared in these proembryos; some cells displayed clear cytoplasm with very large vacuoles and numerous small vesicles and vacuoles (Fig. 1c). Other cells showed dense cytoplasm containing abundant ribosomes and organelles, the nuclei were large and located in the centre. Chromatin was condensed in small patches, one or two nucleoli were observed per nucleus (Fig. 1 c,d).

The formation and differentiation of the cell walls is a key feature in plant developmental processes. Structure and composition of walls was studied during microspore embryogenesis. PTA ultrastructural cytochemistry for polysaccharides (Testillano *et al.*, 1995b) revealed the polysaccharidic component of cell walls of different thicknesses in barley microspore proembryos (Fig. 1e). Immunogold labelling with JIM7 and JIM5 antibodies (Knox *et al.*, 1990) recognizing esterified and non-esterified pectins respectively was performed on multicellular proembryos (Ramírez *et al.*, 2001). Immunocytochemical results revealed differences in the composition of walls. JIM5 antibody provided immunolabelling to the peripheral layer below the exine which was surrounding the proembryo whereas no labelling was observed on the walls separating cells of the proembryo (Fig. 1f). Gold particles localizing esterified pectins, JIM7 antibody, decorated both peripheral and inner cell walls of the proembryo, labelling was specifically localized in the central layer of these walls, probably corresponding with the lamella. (1f). This data revealed the differential composition of inner walls and the peripheral wall located under the exine. These differences between microspore and proembryo walls could represent cellular markers of the process.

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### References

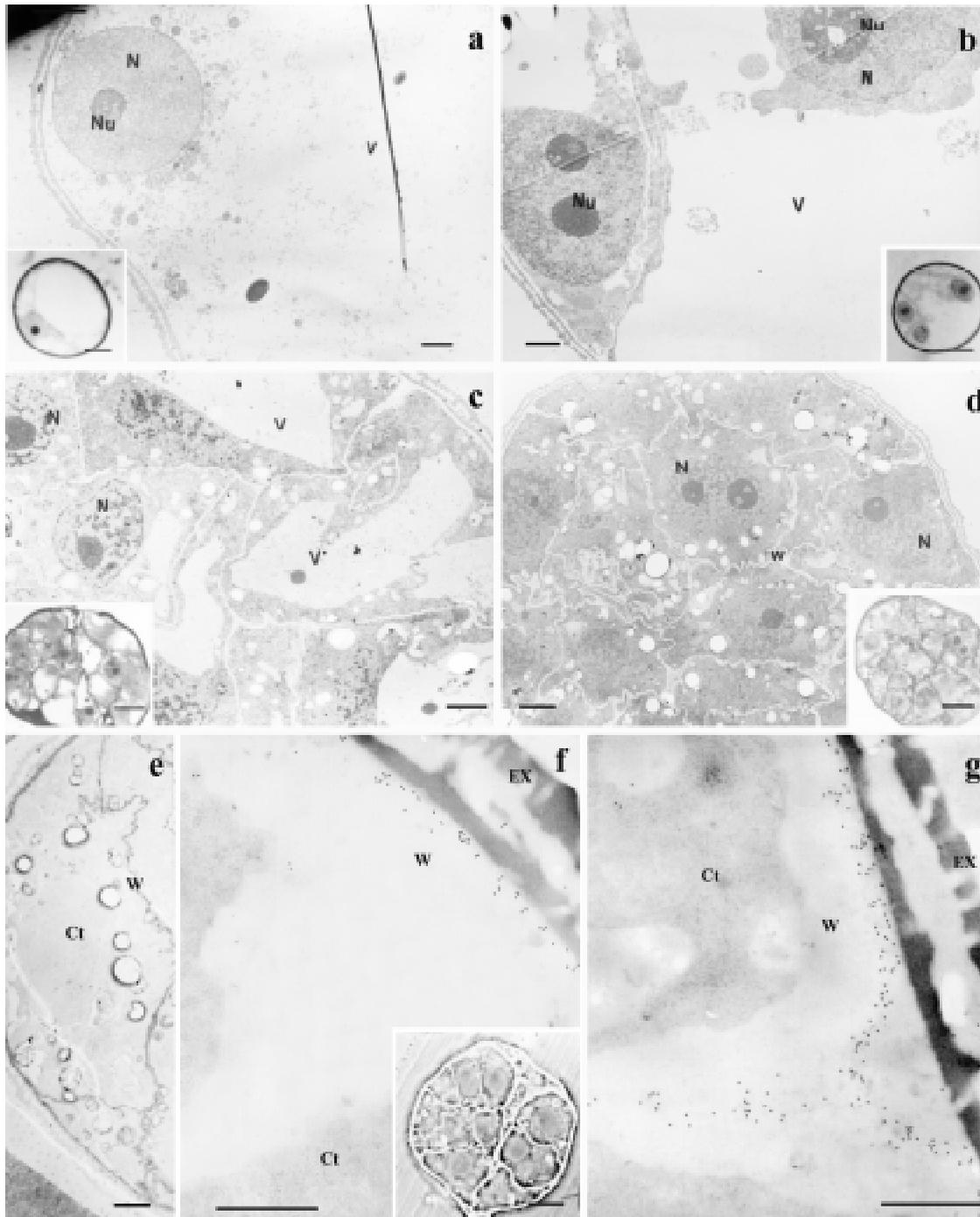
- CISTUÉ, L., RAMOS, A., CASTILLO, A.M. and ROMAGOSA, I. (1994). Production of large number of doubled haploid plants from barley anthers pretreated with high concentrations of mannitol. *Plant Cell Rep* 13:709-712.
- GONZÁLEZ-MELENDE, P., TESTILLANO, P.S., AHMADIAN, P., FADÓN, B. and RISUEÑO, M.C. (1996). Newsitu approaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. *Eur. J. Cell Biol.* 69: 373-386.
- KNOX, J.P., LINDSTEAD, P.J., KING, J., COOPER, C. and ROBERTS, K. (1990). Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* 181: 512-521.

RAMÍREZ, C., TESTILLANO, P.S., CASTILLO, A.M., VALLÉS, M.P., CISTUÉ, L. and RISUEÑO, M.C. (2001). Cellular changes occur during early microspore embryogenesis producing two different regions in barley and wheat. *Submitted*.

TESTILLANO, P.S., GONZÁLEZ-MELENDI, P., AHMADIAN, P., FADÓN, B. and RISUEÑO, M.C. (1995)a. The immunolocalization of nuclear antigens to study the pollen developmental program and the induction of pollen embryogenesis. *Exp. Cell Res* 221: 41-54.

TESTILLANO, P.S., FADÓN, B. and RISUEÑO, M.C. (1995)b. Ultrastructural localisation of the polysaccharidic component during the sporoderm ontogeny of the pollen grain of *Scilla peruviana* L. (Liliaceae) and *Capsicum annuum* L (Solanaceae). *Rev. Paleobot. Palynol.* 85: 53-62.

TESTILLANO, P.S., CORONADO, M.J., SEGUÍ, J.M., DOMENECH, J., GONZÁLEZ-MELENDI, P., RASKA, I. and RISUEÑO, M.C. (2000). Defined nuclear changes of the microspore accompany its reprogramming to embryogenesis. *J. Struct. Biol.* 129: 223-232.



**Fig. 1. Early microspore embryogenesis in barley. (a-d)** Ultrastructure of vacuolate microspore (a) at the beginning of culture, 3-4 cell proembryo (b) and multicellular proembryos (c,d). Insets: Cellular organization of the same samples observed under phase contrast. **(e)** PTA ultrastructure for polysaccharides. **(f,g)** Immunogold labelling with

JIM5 antibody recognizing esterified pectins (f) and JIM7 antibody to esterified pectins (g) in microspore-derived multicellular proembryos. N, nucleus; CT, cytoplasm; V, vacuole; W, cell wall; EX, exine. IR, interchromatin region; CHR, condensed chromatin. Bars in a-e, 2  $\mu$ m; in f,g, 0.5  $\mu$ m, and in insets, 10  $\mu$ m.