CELLULAR CHARACTERIZATION OF KEY DEVELOPMENTAL STAGES FOR POLLEN EMBRIOGENESIS INDUCTION

Pablo GONZÁLEZ-MELENDI[1], Pilar S. TESTILLANO[2], Guadalupe PRÉSTAMO[1], Begoña FADÓN[1], Mª del Carmen RISUEÑO[1]
[1]*Centro de Investigaciones Biológicas, CSIC, Velazquez 144, 28006 Madrid. Spain. Fax: 34-1-562.75.18*
[2]*Centro de Biología Molecular Severo Ochoa, CSIC/UAM, Cantoblanco, 28934 Madrid.*

The potential of pollen grains to deviate from the gametophytic developmental pathway to embryogenesis has been widely used to obtain haploid plants for breeding purposes. In the last years investigations have been focused on the determination of factors involved in the switch to the sporophytic pathway. The developmental stage in which the pollen grain can deviate to embryogenesis, under specific stress conditions is one of the key factors to obtain a positive response to the induction. Vacuolate microspores and bicellular pollen grains have been described as suitable periods to induce embryogenesis; a more precise characterization of the developmental stage in which the cell is able to change its program would help to understand the process and select more efficiently the microspore populations for the induction.

We have induce pollen embryogenesis in *Capsicum annuum* L, pepper. Multicellular pollen grains (Figs. 2-5) were obtained in *in vitro* cultures of isolated microspores after stress treatments of semistarvetion and/or heat shock at 37°C. As material to begin the *in vitro* culture, anthers containing vacuolate microspores and early bicellular pollen grains (Fig. 1) showed better response to the embryogenesis induction. The most responsive cell population seemed to be the late vacuolate microspores (Fig. 6) as indicated by the analysis of the cell division pathway followed during the embryogenic culture. Various *in situ* methods including cytochemistry, immunocytochemistry, and *in situ* hybridization have been used to characterize the functional organization of the microspore nucleus at this specific developmental stage in which the change from the gametophytic to the sporophytic program can be induced. This cellular study would enable to characterize the physiological state of sensitive phases for embryogenesis induction and provide data to stablish cellular markers of the process of pollen embryogenesis. Material processed at low temperature (cryosections and Lowicryl sections) were used for immunofluorescence, immunogold labelling and *in situ* hybridization purposes.

Cell cycle-dependent events have been reported to be involved in pollen embryogenesis induction in other plant system, the entry in S phase being necessary for the switch of the developmental program in the vegetative cell of the bicellular pollen grain. Immunolocalization of the Proliferating Cell Nuclear Antigen (PCNA), the auxiliary protein of the DNA pol δ, has been performed in vacuolate microspores and bicellular pollen grains to study replication sites and S phase progresion. The pattern of PCNA labelling was different at both developmental stages, the results revealing that late vacuolate microspore is in a replicative state whereas the two nuclei of the young bicellular pollen grain do not. A quantitative study showed the existence of two distributions of PCNA labelling: clusters of gold particles at the periphery of condensed chromatin masses (Fig. 7), corresponding to replication complexes, and scarce isolated particles dispersed in the nucleoplasm, which probably represent the population of soluble PCNA not associated to replication sites.

Participation of MAP kinases (MAPk) pathway in either the stress-signal transduction triggering pollen embryogenesis or the switch to proliferation of *in vitro* cultured microspores has been suggested in some plant systems. RNA probes and antibodies recognizing MAPk mRNAs and proteins were used to study the presence and distribution of both molecular targets during pollen development. Differences in labelling density and subcellular location will be studied on the ligth of their relation to specific cell cycle events and the susceptibility of some pollen developmental periods to embryogenesis induction.

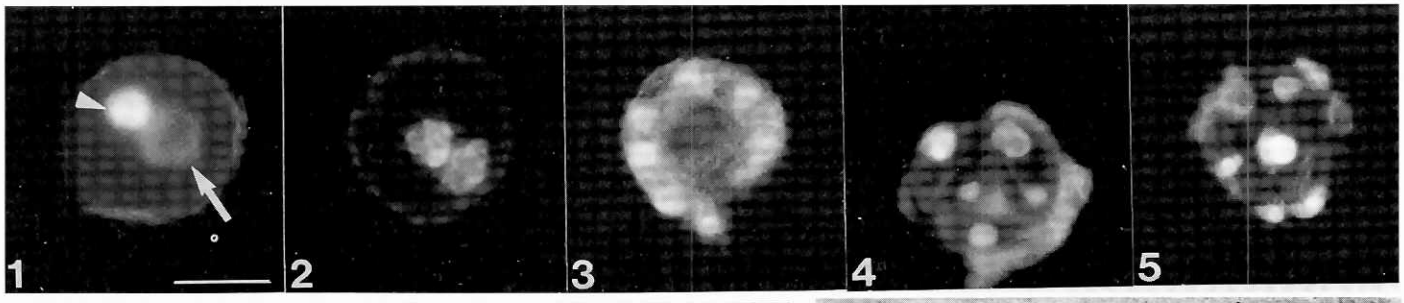
Other *in situ* studies have been performed to characterize the state of nuclear activity in the embryogenesis-responsive developmental stage of vacuolate microspore. *In situ* Terminal deoxy-nucleotidyl Transferase (TdT) technique for DNA and specific ultrastructural cytochemistry methods revealed a characteristic pattern of chromatin displaying abundant decondensed chromatin areas (Fig. 8). Anti-snRNPs antibodies showed splicing factors on specific interchromatin structures in the vacuolate microspore nucleus. Immunogold labelling with anti-DNA/RNA hybrid antibodies localized sites of transcription at the perichromatin area and regions of the nucleolar dense fibrillar component, revealing a high transcription activity. Various antigens of the pre-rRNA synthesis and processing machinery (UBF, fibrillarin, total RNA...) have been also immunolocalized in the late vacuolate microspore nucleus, displaying characteristic patterns of distribution. Ultrastructural *in situ* hybridization using 18S and 25S ribosomal probes provided data on the distribution of ribosomal transcripts in the nucleus of the pollen grain at the specific developmental stages in which embryogenesis can be induced. Differences on the distribution of rRNA in different nucleolar components have been observed in relation to transcriptional activity rate and developmental stage.

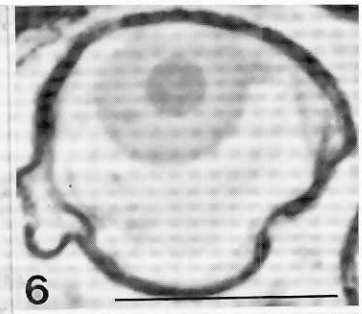
The *in situ* data enabled the cellular characterization of the phase of late vacuolate microspore as a highly active period of the haploid genome during pollen development. The immunogold and *in situ* hybridization approach used have enabled the study of chromatin pattern, DNA replication, extranucleolar transcription and processing and ribosome biogenesis at both late vacuolate microspore and young bicellular pollen grain periods, revealing in the former a physiological state which could probably be related to a higher embryogenic response in *Capsicum*. The cellular features displayed by the vacuolate

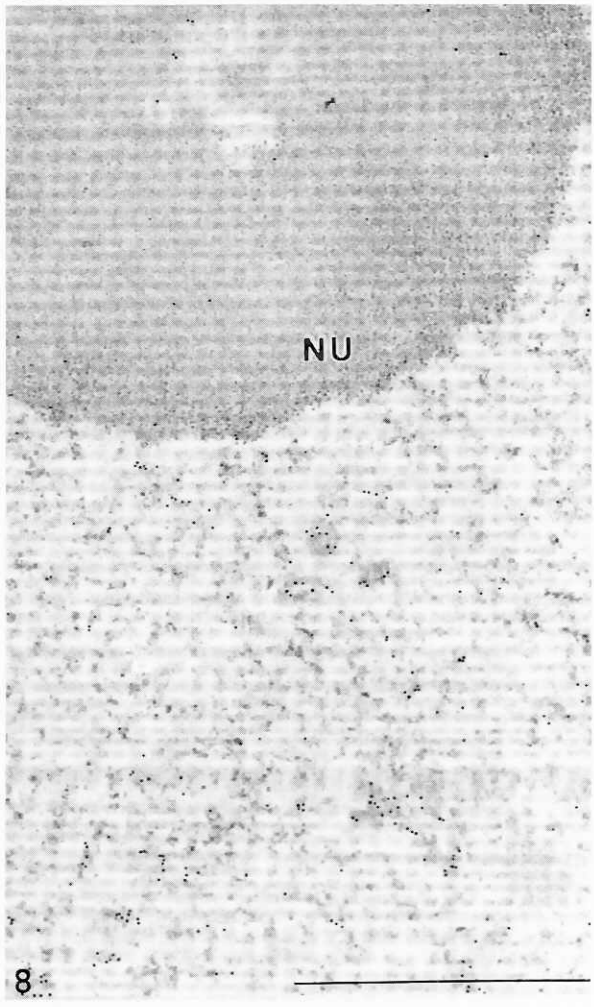
microspore *in vivo* and their comparison with embryogenic pollen will shed ligth on the biochemical pathway triggering pollen embryogenesis.

References

González-Melendi , P., Testillano, P.S., Ahmadian, P., Fadón, B., Vicente, O. and risueño, M.C, (1995) *In situ*characterization of the late vacuolate microspore as a convenient stage to induce embryogenesis in *Capsicum*.Protoplasma 187: 60-71.
Testillano, P.S., González-Melendi, P., Ahmadian, P., and Risueño, M.C. (1995). The immunolocalization of nuclear antigens to study the pollen embryogenesis. Exp. Cell Res.221: 41-54.
González-Melendi, P., Testillano, P.S., Ahmadian, P., Fadón, B. and Risueño, M.C. (1996). New *in situ* aproaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. Eur. J. Cell Biol. 69: 373-386.







Figs. 1 to 5: DAPI staining for DNA. Fig. 1: *In vivo* development, bicellular pollen grain. Vegetative (arrow) and generative (arrowhead) nuclei show different fluorescence intensity. Figs. 2, 3, 4 and 5: Embryogenic culture. Bicellular and multicellular pollen grains developped *in vitro* . Figs. 6, to 8: Late vacuolate microspore. Fig. 6: Ligth micrograph under phase contrast . Fig. 7: PCNA immunogold labelling. Clusters (arrows) of gold particles appear in the interchromatin region (IR). Fig. 8: *In situ* TdT reaction , labelling decorates small chromatin patches and fibres. Nu, nucleolus. Bars in LM micrographs represent 10 µm, in EM micrographs 1 µm.