

**IN VIVO LABELING OF SUNFLOWER (*HELIANTHUS ANNUUS* L.)  
EMBRYONIC TISSUES BY FLUORESCENT PHENYLALKYLAMINE  
HIGHLIGHTS THE EMBRYO PROTODERM ROLE IN ION  
EXCHANGES VIA ION CHANNELS\***

**Xu XuHan<sup>1,2</sup>, Christian Brière<sup>1</sup>, Nathalie Vallée<sup>1</sup>, Gilbert Alibert<sup>1</sup> and André Souvré<sup>1</sup>,**  
<sup>1</sup>Laboratoire de Biotechnologie et Amélioration des Plantes, INP-ENSAT-UA INRA, Avenue  
de l'Agrobiopole, BP 107 Auzeville-Tolosane, 31326 Castanet-Tolosan, France  
<sup>2</sup>IRIT-ARI, 202 bis rue des Fontaines, 31300 Toulouse, France  
Fax: +33 05 62 19 35 89; e-mail: briere@ensat.fr

**Summary :** Cytological structure and ion channel distribution in sunflower zygotic and somatic embryos, somatic tissues and callus were investigated. A protoderm formed at the end of the proglobular embryo stage, in both zygotic embryos and somatic embryos. The protoderm cells showed a highly cytoplasmic-rich character compared with the inner embryonic cells and callus cells, which were more vacuolated. Both cytofluorometry and confocal microscopy revealed differential labeling patterns of DM-Bodipy PAA (phenylalkylamine) in embryonic and non-embryonic tissues. DM-Bodipy PAA intensively labeled the protoderm and epidermis cells in both zygotic and somatic embryos. The callus exhibited labeling on sites where somatic embryos developed. Labeling was very weak in leaves, shoots and roots, except in the root cap and in the epidermis of the root. Considering that the location of phenylalkylamine binding sites is related to K<sup>+</sup>- and especially Ca<sup>2+</sup>-channels in both animal and plant cells, and that high labeling levels were observed in root tip and epidermis, the high intensity of labeling observed in the protoderm and epidermis of zygotic and somatic embryos points to the function of ion exchanges via ion channels.

**Keywords:** *Helianthus annuus*, embryo, epidermis, protoderm, phenylalkylamine, ion channel

---

\* Part of this work is published in XuHan et al. *Protoplasma* **210** : 52-58 (1999)

## INTRODUCTION

Higher plant tissues at the interface between the plant body and the external medium are essential sites for communication between plants and the environment, which calls attention to the functions of the plant epidermis system, especially in signal transduction pathways. To date, it is well known that ion channels play an important role in signal transduction and are also involved in the control of morphogenesis in plants (Ward et al. 1995; Cho and Hong 1996). A fluorescently labeled phenylalkylamine (DM-Bodipy PAA) that blocks the L-type  $\text{Ca}^{2+}$ -channel activity in animal cells (Norris and Bradford 1985) has been used as a probe for labeling  $\text{Ca}^{2+}$ -channel antagonist binding sites in animal cell membranes (Knaus et al. 1992). And recently, this probe was used to label phenylalkylamine (PAA) binding sites in sunflower protoplasts (Vallée et al. 1997, Vallée et al. 1999) and embryos (XuHan et al. 1999). In the present work we used electron microscopy to compare sunflower zygotic and somatic embryos, to identify *in vitro* embryonic tissue structure, and used DM-Bodipy PAA to study the distribution of PAA binding sites with confocal microscopy. Special attentions are paid to the epidermis system.

## MATERIALS AND METHODS

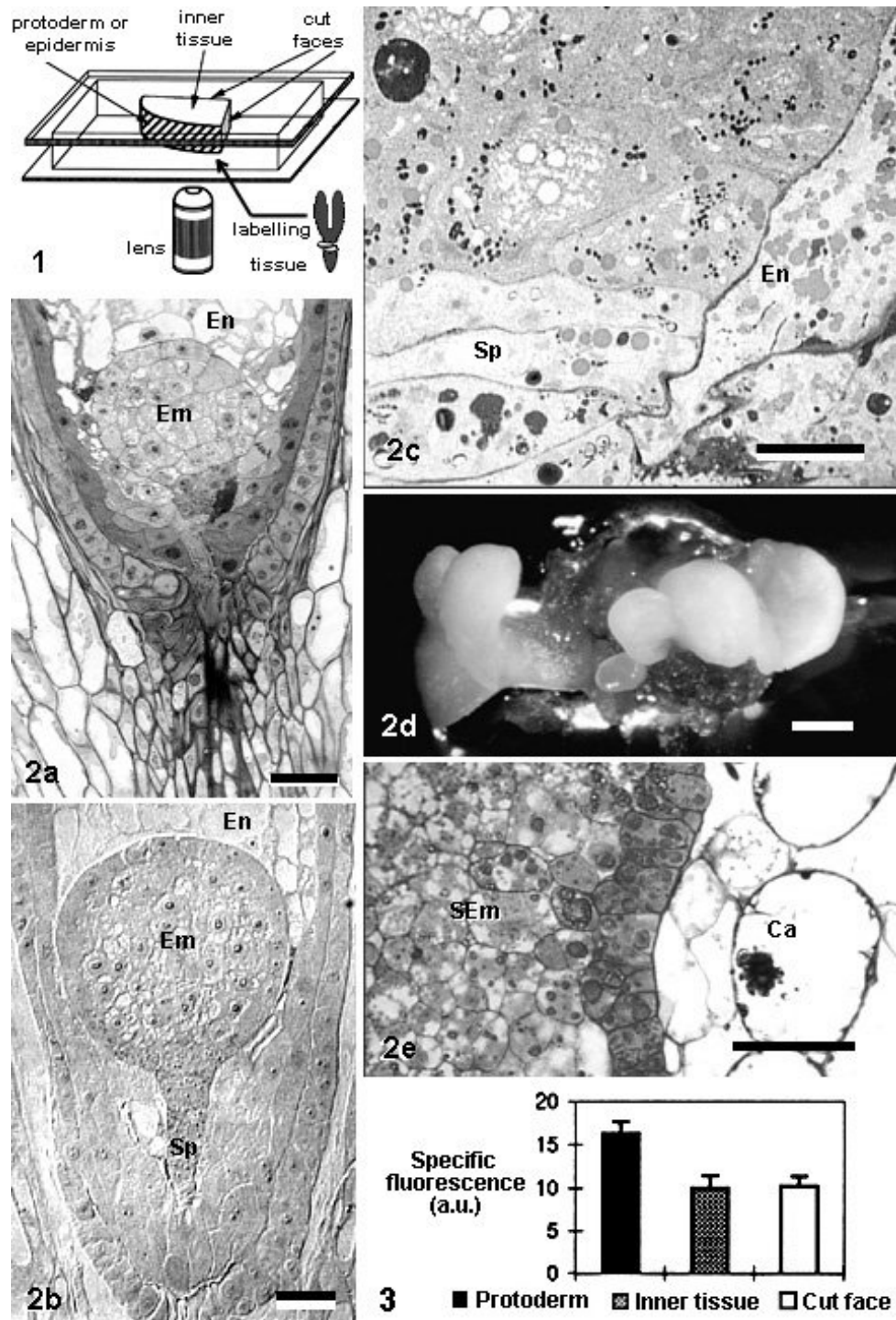
**Plant materials** Sunflower (*Helianthus annuus* L. genotype HA300B) zygotic embryos at different stages were isolated. Mature zygotic embryos were germinated in a culture medium containing MS salts (Murashige and Skoog 1962). The embryos, leaves, shoot apices and roots were collected for investigations. *In vitro* culture of immature zygotic embryos was performed by placing heart to late cotyledonary embryos on a modified MS medium to induce uneven callogenesis. Somatic embryogenesis was induced on peeled strips of the hypocotyl, according to Pélissier *et al.* (1990).

**Light and electron microscopy** Tissue samples were fixed in 3% paraformaldehyde and 0.5%  $\text{OsO}_4$ , both in PBS puffer at pH 7.0. After dehydration, the samples were embedded in a Spurr resin. Semithin sections and ultrathin sections were stained and observed by conventional light microscope and electron transmission microscope.

**Probe loading and controls** A  $\text{Ca}^{2+}$ -channel probe DM-Bodipy PAA, and a  $\text{Ca}^{2+}$ -channel antagonist (-)-Bepriidil, were used. Embryos, leaves, shoot apices and calli, obtained from both zygotic and somatic pathways, were hand-cut in approximately 0.5-1 mm thick sections. These samples, and whole root tips, were successively incubated in 1  $\mu\text{M}$  DM-Bodipy PAA solution at 25°C in Eppendorf tubes for at least 30 min, rinsed in MS-120 medium, and mounted in an observation chamber (**Fig. 1**). Samples incubated in MS-120 medium without DM-Bodipy PAA were used as controls of autofluorescence. Samples incubated in MS-120 medium with unconjugated Bodipy (Bodipy FL) were used as control for the specific binding. Specificity of DM-Bodipy PAA labeling was determined by measuring fluorescence on the same site after washing samples in 100 $\mu\text{M}$  (-)-Bepriidil and MS-120 medium.

**Quantitative image analysis of fluorescence** A Leitz orthoplan microscope (Weitzlar, Germany) was used. Acquisition time and settings of microscope and camera were maintained the same for all measurements. Fluorescence images were analyzed in three zones, *i.e.* the outer layers with protoderm or epidermis, the inner tissue part, and the inner tissue near the cut edge (Ref. **Fig. 1**). In statistical comparison, standard analysis of variance and Sheffe's multiple comparison procedures (risk level  $\alpha = 0.05$ ) were used for in total 80 embryo transverse sections.

**Confocal Laser Scanning Microscopy (CLSM)** A confocal laser scanning microscope (Zeiss LSM 410 Microsystem, Germany) equipped with an Argon-ion laser (excitation at 488 nm with a 510-525 nm emission filter) was used to analyze DM-Bodipy PAA labeling.



**Fig. 1.** Schematic representation of tissue preparation for tissue samples and measurement in transverse sections in an observation chamber. **Fig. 2.** a) Sunflower zygotic embryos (a – c) and somatic embryos (d – e). A zygotic embryo (Em) at transition from the proglobular stage to globular stage, when the protoderm formed. Bar = 20  $\mu\text{m}$ . b) Zygotic embryo at globular stage. The suspensor (Sp) elongated in the micropylar end. Bar = 20  $\mu\text{m}$ . c) Electron micrograph showing a part of a globular embryo. Note the close contact between the embryo and the endosperm tissue (En). Bar = 5  $\mu\text{m}$ . d) Somatic embryos developed on the callus derived from the original hypocotyl epidermal layers cultured *in vitro*. Bar = 5 mm. e) Somatic embryo (SEm) which showed a highly cytoplasmic-rich character compared with the adjacent, very vacuolated callus cells (Ca) and the inner embryonic cells. Bar = 20  $\mu\text{m}$ . **Fig. 3.** Fluorescence intensities of DM-Bodipy PAA labeling in the protoderm, inner tissue and cut face of transverse sections of zygotic embryos. Data represent the mean  $\pm$  s.e. of 80 sections.

## RESULTS

### Development and structure of protoderm

From the third day to the fourth day after anthesis, a protoderm was formed on the outermost layer of the globular embryo (**Fig. 2a**). During embryogenesis, embryonic protoderm cells remained cytoplasmic-rich for a longer time than the internal cells (**Fig. 2b**). The suspensor at the embryo basal end, elongated in the micropylar part (**Fig. 2b**). At ultrastructural level, the suspensor cells were found less cytoplasmic than the embryo proper cells (**Fig. 2c**). In somatic embryogenesis, somatic embryos were induced from hypocotyl epidermal layer cultures. The embryos usually developed on the superficial part of the callus derived from original hypocotyl epidermal layers (**Fig. 2d**). A protoderm was also formed on the embryos, and it was the cell layer with the denser cytoplasm, and the callus cells were most vacuolated (**Fig. 2c**). No protoderm-like structures were observed on the callus itself.

### Quantification of DM-Bodipy PAA labeling in zygotic embryos

When loaded with DM-Bodipy PAA, the tissues of the zygotic embryos showed significantly higher fluorescence intensity than the autofluorescence level. Statistical comparison of the mean fluorescence measured in various parts of the sections revealed that the fluorescence in the outer cell layers was significantly stronger than that in cells of the inner tissues, even when the inner tissues were the borders of the radial cut-face (Ref. **Fig. 1**). In all parts of the samples the probe-induced fluorescence signal was significantly reduced by (-)-Bepridil, but remained higher than the autofluorescence level. An estimate of DM-Bodipy PAA specific fluorescence was obtained by subtracting the mean fluorescence measured after incubation with (-)-Bepridil from the mean fluorescence measured before (-)-Bepridil treatment (**Fig. 3**).

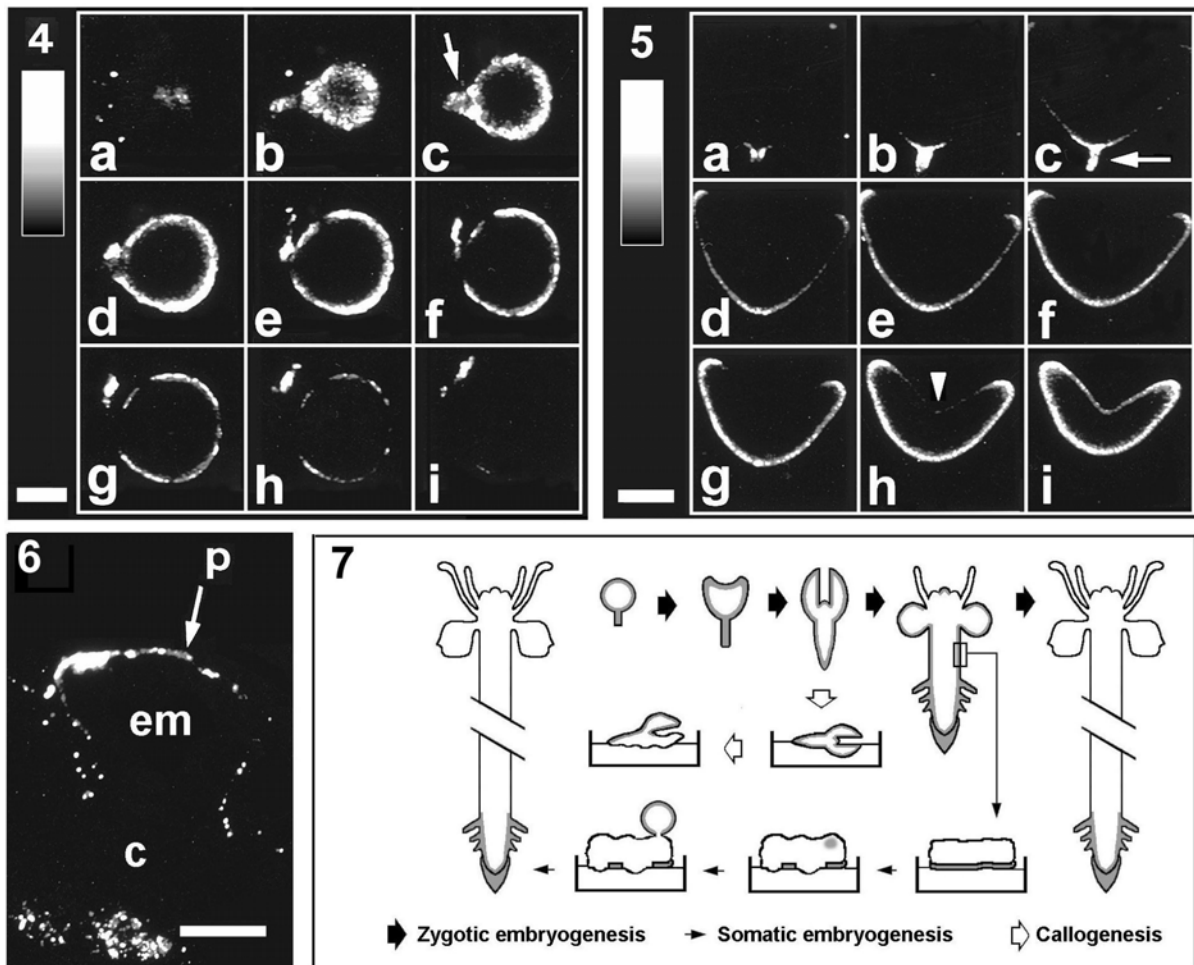
### Confocal analysis of embryonic and non-embryonic tissues

**Zygotic embryos and germlings** The protoderm and the suspensor of the zygotic embryos showed a strong DM-Bodipy PAA signal from the globular stage and onwards (**Figs. 4 - 5**). When whole mount embryos were analyzed by CLSM, signals were often faint, due to the thickness of the embryo proper (see *e.g.* **Figs. 4 h, i** and **5**). In mature embryos, the protoderm or epidermis of cotyledons and hypocotyl showed also a strong labeling. After germination, the labeling in the outermost cell layers disappeared. Leaf primordia and the shoot apex did not exhibit DM-Bodipy PAA labeling either. In contrast, in the root tips, the root cap, the protoderm without root hairs, and the epidermis with root hairs were strongly. The cell layers covered by the root cap also showed a strong signal.

**In vitro cultured zygotic embryos** In immature zygotic embryos cultured *in vitro*, the lower cotyledon, which was in direct contact to the culture medium, lost its smooth epidermis, enlarged and became callogenic. The embryo axis and the upper cotyledon kept the normal developmental pattern of germination and still exhibited DM-Bodipy PAA labeling at their outer cell layers. The cells at the periphery of the callus were not labeled.

**Callus and somatic embryos** In cultured epidermal strips, the original epidermis, which attached to the callogenic subepidermal tissue, showed a strong labeling. Most callus cells showed no or only a very weak fluorescence, except in the areas where somatic embryos were to be formed. Calli which did not develop protoderm and epidermis, did not show labeling.

When somatic embryos developed further, they showed a fluorescence pattern similar to that of zygotic embryos: a signal was only observed in the outermost cell layers (**Fig. 6**).



**Fig. 4.** Series of optical sections of an intact zygotic embryo at the globular stage labeled with DM-Bodipy PAA and observed by CLSM. Note the strong labeling in the outer cell layers and the suspensor (arrow). Bar = 100  $\mu\text{m}$ . **Fig. 5.** Series of optical sections of an intact zygotic embryo at the heart-shaped stage labeled with DM-Bodipy PAA as observed by CLSM. The outer cell layers and the suspensor (arrow) are strongly labeled. Bar = 200  $\mu\text{m}$ . **Fig. 6.** Confocal optical section of a somatic embryo on callus. Note the intensive DM-Bodipy PAA labeling of the protoderm (p). (em) embryo, (c) callus. Bar = 100  $\mu\text{m}$ . **Fig. 7.** Schematic representation of DM-Bodipy PAA labeling (grey shades) in embryos and plants of sunflower during the development of zygotic embryos to plants ( $\blacktriangleright$ ), during the culture of zygotic embryos ( $\Rightarrow$ ), and during the culture of hypocotyl strips giving rise to somatic embryos ( $\rightarrow$ ).

## DISCUSSION

Fluorescent probes provide useful tools to study cytomorphogenesis during embryo development *in vivo* and *in vitro* (XuHan *et al.* 1995; Timmers *et al.* 1996). Combined with structural analysis, we defined a particular binding region of DM-Bodipy PAA in sunflower tissues. Because PAA binds to plasma membranes (Vallée *et al.* 1997), inhibits  $\text{Ca}^{2+}$  influx or blocks  $\text{Ca}^{2+}$ -channel activity (Piñeros and Tester 1997), binds to isolated  $\text{Ca}^{2+}$ -channel peptides, and affects the activity of  $\text{K}^{+}$ -selective channels (Terry *et al.* 1992), its binding sites in plant tissues seem highly relate to the  $\text{K}^{+}$ - and especially  $\text{Ca}^{2+}$ -channel sites. As summarised in **Fig. 7**, strong DM-Bodipy PAA labeling signals have been recorded in the suspensor, the protoderm and the epidermis of both zygotic and somatic embryos, but a weak

fluorescence was observed in the inner embryonic tissues and also in non-embryonic tissues with the exception of the root tips. The weak labeling is considered to represent a low density of PAA binding sites. The similarities in the labeling pattern found in the zygotic and somatic embryos point to similar properties and functions of their epidermal cells. The strong labeling of the suspensor of the zygotic embryo of sunflower can be related to the critical role played by this structure during early embryogenesis, e.g. the transport of nutrients and growth regulators (Yeung and Meinke 1993). The labeling of the protoderm of the zygotic embryo could be linked to the transfer of nutrients and signalling molecules from the endosperm or maternal tissues to the embryo proper. The differences between the labeling of the root and the shoot apex further indicate the function of the tissue bearing ion channels in the light of ion exchange. It is likely that there are two types of epidermis in sunflower plant, one not labeled and functioning in protection, the other one intensively labeled and probably also functioning in interaction with the environment by the exchange of ions.

## References

- Cho HT, Hong YN (1996) Effect of calcium channel blockers on the IAA-induced cell elongation of sunflower hypocotyl segments. *J. Plant Physiol.* 149: 377-383
- Knaus HG, Moshammer T, Kang HC, Haugland RP, Glossman H (1992) A unique fluorescent phenylalkylamine probe for L-type  $Ca^{2+}$ -channels. *J. Biol. Chem.* 267: 2179-2189
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15 : 473-497
- Norris DK, Bradford HF (1985) On the specificity of verapamil as a calcium channel blocker. *Biochem. Pharmacol.* 34: 1953-1956
- Péllissier P, Boucheffa O, Pépin R, Freyssinet G (1990) Production of isolated somatic embryos from sunflower thin cell layers. *Plant Cell Rep* 9 : 47-50
- Piñeros M, Tester M (1997) Characterisation of the high-affinity verapamil binding site in a plant plasma membrane  $Ca^{2+}$ -selective channel. *J Membrane Biol* 157 (2): 139-145
- Terry BR, Findlay GP, Tyerman SD (1992) Direct effects of  $Ca^{2+}$ -channel blockers on plasma membrane cation channels of *Amaranthus tricolor* protoplasts. *J Exp Bot* 43 : 1457-1473
- Timmers ACJ, Reiss HD, Boshung J, Traxel K, Schel JHN (1996) Localization of calcium during somatic embryogenesis of carrot (*Daucus carota* L.). *Protoplasma* 190 : 107-118
- Vallée N, Brière C, Petitprez M, Souvré A, Alibert G (1997) Studies on ion channel antagonist-binding sites in sunflower protoplasts. *FEBS Lett* 411 : 115-118
- Vallée N, Brière C., Petitprez M, Barthou H, Souvré A, Alibert G (1999) Cytolocalization of ion-channel antagonist binding sites in sunflower protoplasts during the early steps of culture. *Protoplasma* 210: 36-44
- Ward JM, Pei Z-M, Schroeder JI (1995) Roles of ion channels in initiation of signal transduction in higher plants. *Plant Cell* 7: 833-844
- XuHan X, Brière C, Vallée N, Borin C, Van Lammeren AAM, Alibert G, Souvré A (1999) In vivo labeling of sunflower embryonic tissues by fluorescently labeled phenylalkylamine. *Protoplasma* 210: 52-58
- XuHan X, Souvré A, Granier C, Petitprez M (1995) An improved immunolabeling method for microtubular cytoskeleton in poplar free nuclear endosperm. *Biotech Histochem* 70: 252-257
- Yeung EC, Meinke DW (1993) Embryogenesis in angiosperms: development of the suspensor. *Plant Cell* 5 : 1371-1381