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In vivo labeling of sunflower embryonic tissues by fluorescently labeled phenylalkylamine

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Summary. A fluorescently labeled phenylalkylamine, DM-Bodipy PAA, was used as a probe for the in vivo detection of ion channels in embryonic and nonembryonic tissues of sunflower. Zygotic embryos, somatic embryos, callus, leaves, roots, and shoots were analysed. Fluorescence intensity in the tissues was determined with cytofluorometry and confocal microscopy. DM-Bodipy PAA intensively labeled the protoderm and epidermis cells in both zygotic and somatic embryos. Callus cultures exhibited labeling on sites where somatic embryos developed. Labeling was, however, 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 the distribution of ion channels in both animal and plant cells, the high intensity of labeling observed in the protoderm and epidermis of zygotic and somatic embryos as well as in protoderm, epidermis, and caps of root tips, is consistent with the role these tissues may play in ion exchange with the environment.

Keywords: *Helianthus annuus*; Embryo; Epidermis; Protoderm; Phenylalkylamine; Ion channel.

Introduction

Tissues which are at the interface between the plant body and the external medium are essential sites for communication between plant and environment. This is particularly crucial for the protoderm and the epidermis of young embryos. Recent investigations have revealed that specific genes are expressed during the development of embryonic protoderm and epidermis (Sterk et al. 1991, Vroemen et al. 1996); embryonic protoderm shows a calcium binding pattern which differs from that in the inner embryonic cells (Timmers et al. 1996); and embryonic protoderm cells are adjacent to sites where endosperm digests specifically (XuHan and Van Lammeren 1997). These findings call the attention upon the functions of the plant epidermis system, especially in signal transduction pathways. Although 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), their distribution in higher-plant tissues has not been described yet. Phenylalkylamines (PAAs) are pharmacological drugs known to block specifically the L type Ca^{2+}-channel activity in animal cells (Norris and Bradford 1985). A fluorescently labeled PAA, DM-Bodipy PAA, has been used as a probe for labeling Ca^{2+}-channel antagonist binding sites in order to localise Ca^{2+}-channels in isolated animal membranes (Knaus et al. 1992). Recently, this probe was used to label PAA binding sites in sunflower protoplasts (Vallée et al. 1997). Although Ca^{2+}-channel-active drugs may not be highly specific in plant cells (Terry et al. 1992, Thomine et al. 1994), PAA has been shown to block calcium to enter into plant cells (Andrejauskas et al. 1985, Graziana et al. 1988, Harvey et al. 1989, Thuleau et al. 1990). Thus, DM-Bodipy PAA appears to be a good tool to study the distribution of Ca^{2+}-channel antagonist binding sites in higher-plant tissues.

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In the present work we have used DM-Bodipy PAA to study the distribution of PAA binding sites in sunflower tissues and paid special attention to the epidermis systems of zygotic embryos, somatic embryos, and nonembryonic explants.

Material and methods

Plant materials

Sunflower, *Helianthus annuus* L., genotype HA300B, was grown in the greenhouse (Novartis Seeds, St. Sauveur, France). Zygotic embryos were isolated manually from ovaries at different stages of postfertilisation development to obtain globular to mature stages. Mature zygotic embryos were germinated in the dark in culture medium containing MS salts (Murashige and Skoog 1962). Leaves, shoot apexes, and roots were collected for investigations.

In vitro culture of immature zygotic embryos was performed by incubating heart to late cotyledonary embryos in MS medium (Murashige and Skoog 1962) solidified with Phytagel (Sigma, St. Louis, Mo., U.S.A.) and supplemented with 120 g of sucrose, 1 mg of 6-benzylaminopurine (BAP) and 1 mg of α-naphthaleneacetic acid (NAA) per ml (MS-120BN), in darkness for 8 days. The embryos were longitudinally placed on the medium in a way that one half of the embryo with a cotyledon was in direct contact with the medium while the other side had no contact with the medium. Under these culture conditions, the lower cotyledon became callogenic while the embryo axis elongated; the upper cotyledon retained its normal embryonic development.

Somatic embryogenesis was induced on peeled strips of the hypocotyl. The strips which included the epidermis and about 4 subepidermal cell layers were cultivated according to Pélissier et al. (1990).

Chemicals

Fluorescently labeled PAA, DM-Bodipy PAA (5-[3-[3-(4,4-difluoro-5,7-dimethyl-3a,4a-diaza-4-bora-indacen-3-yl)propionamido] phenethyl-N-methylamino]-2-isopropyl-2-(3,4,5-trimethoxy-phenyl)-valeronitrile) (Molecular Probes, Eugene, Oreg., U.S.A.) was prepared as a 2.5 mM stock solution in dimethyl sulfoxide (DMSO; Sigma).

(−)Bepridil, a calcium channel antagonist (a gift from Dr. Ranjeva, CNRS/UPS, Toulouse, France), was prepared as a 20 mM stock solution in ethanol.

Probe loading

Before use, the DM-Bodipy PAA stock solution was diluted to 1 mM in MS medium (Murashige and Skoog 1962) supplemented with 120 g of sucrose per ml (MS-120).

Embryos, leaves, shoot apexes, 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 mM 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).

Controls

Samples incubated in MS-120 medium without DM-Bodipy PAA were used as controls to measure autofluorescence levels.

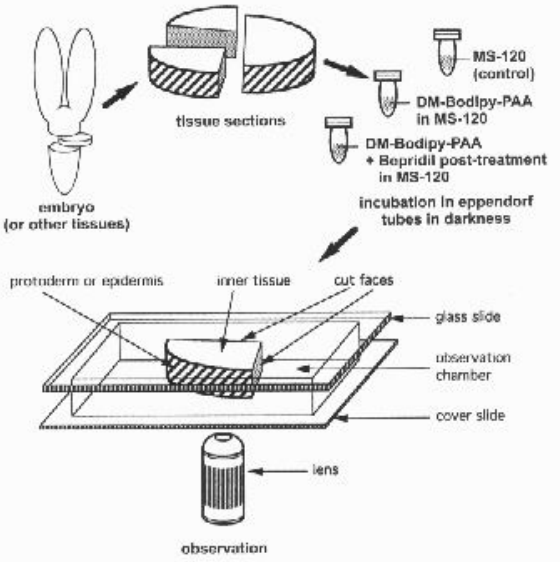


Fig. 1. Schematic representation of tissue preparation for *H. annuus* embryos and probe-loading control. The intensity of fluorescence was measured in transverse sections in an observation chamber. Three embryonal regions were analysed: the protoderm or epidermis, the inner tissues, and the inner tissues next to the longitudinal and radial cut face that could improve accessibility for the probe

The specificity of DM-Bodipy PAA labeling was tested with (−)bepridil which is known to bind to the same sites as PAA and to suppress specific binding of DM-Bodipy PAA in sunflower protoplasts (Vallée et al. 1997). First, the fluorescence of DM-Bodipy PAA labeled explants was measured. Then, the samples were incubated in MS-120 medium containing 100 µM (−)bepridil for 30 min at 25 °C in the darkness after which fluorescence was measured again. The difference of fluorescence was considered as the value of specific binding released by competition. The persisting fluorescence was considered as background due to aspecifically bound DM-Bodipy PAA.

The extent of penetration of the probe into the inner tissues of the samples was tested by comparing the fluorescence level of the tissue at the cut faces to that of the inner tissue. The different parts of the tissue and the overall preparation are shown in Fig. 1.

Quantitative image analysis of fluorescence

A Leitz orthoplan microscope (Leitz, Wetzlar, Federal Republic of Germany), equipped with a 100 W HBP stabilised lamp (Osram, Munich, Federal Republic of Germany) and a silicon intensified (SIT) video camera (Lhesa, Cergy-Pontoise, France) were used for image acquisition. The fluorescence, measured on a grey level scale (range, 0 to 255 arbitrary units), was recorded with a 25/0.55 NPL Fluotar objective (Leitz), with a 450–490 nm wavelength excitation filter, a 510 nm wavelength dichroic mirror, and a 515–525 nm wavelength emission filter (L3 filter cube; Leitz).

DM-Bodipy PAA fluorescence was quantified in zygotic embryos at the heart-shaped to mature stage. From each embryo, 1 to 2 transverse sections were obtained. Each section was cut into 2 to 3 pieces to be used for control and labeling. After recording digital images

of the various regions of the sections, the images were analysed to determine the fluorescence intensities in three zones, i.e., the outer layers with protoderm or epidermis, the inner tissue part, and the inner tissue near the cut edge (see Fig. 1).

For statistical comparison, standard analysis of variance and Sheffe's multiple comparison procedures (risk level, $\alpha = 0.05$) were used for in total 80 pieces obtained from the transverse sections.

Confocal laser scanning microscopy

A confocal laser scanning microscope (CLSM) (Zeiss LSM 410 Microsystem; Zeiss, Oberkochen, Federal Republic of Germany) equipped with an argon ion laser (excitation at 488 nm wavelength with a 510–525 nm wavelength emission filter) was used to analyse the DM-Bodipy PAA labeling in whole mounts or sections of embryos from the globular stage onwards.

For image acquisition a ×25/0.8 Plan Neofluar Zeiss objective was used with a 32% laser attenuation filter (neutral density) to reduce fluorescence bleaching. Each image corresponds to the average of four scanning frames. Samples were also observed without attenuation to detect low fluorescence intensities. In this paper, "no labeling" means that there was no detectable fluorescence signal in the recorded images at standard conditions (i.e., ×25, 32% attenuation filter). Photographs were taken from the digital images with a CONTAX 167MT camera on Kodak Ektachrome Elite ASA 100 films.

Results

Quantification of DM-Bodipy PAA labeling in zygotic embryos

When loaded with DM-Bodipy PAA, the tissues of the zygotic embryos of sunflower showed a fluorescence intensity significantly higher than the autofluorescence level (Fig. 2A). 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 (see 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 (Fig. 2A). In the protoderm the signal was reduced by about 50% and in the inner tissues by about 30%. 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. 2B). Highest specific labeling was observed in the protoderm, whereas low fluorescence was observed in the inner tissue, and no difference was found between labeling of the inner tissues and the cut face region.

Confocal analysis of DM-Bodipy PAA labeling in embryonic and nonembryonic tissues

Zygotic embryos and germlings

The protoderm and the suspensor of developing zygotic embryos showed a strong fluorescence from the globular stage and onwards when loaded with DM-Bodipy PAA (Figs. 3 and 4). When whole mount embryos were analysed by CLSM, signals were often faint, due to the thickness of the embryo proper (see, e.g., Figs. 3h, i and 4 a–g).

In mature embryos, the protoderm or epidermis of cotyledons and hypocotyl showed also a strong labeling by DM-Bodipy PAA. After germination and during further elongation of the shoot, the labeling in the outermost cell layers disappeared. Leaf primordia and the shoot apex did not exhibit DM-Bodipy PAA labeling either.

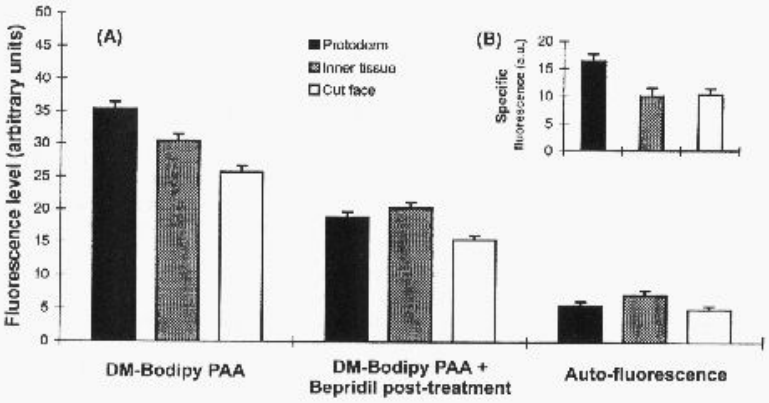


Fig. 2. Determination of fluorescence intensities of DM-Bodipy PAA labeling in protoderm, inner tissue and cut face of transverse sections of zygotic embryos of *H. annuus*. **A** Fluorescence intensities were measured after DM-Bodipy PAA labeling and after DM-Bodipy PAA labeling plus (–)bepridil post-treatment; and the autofluorescence of the tissues was determined (control). **B** Specific labeling by DM-Bodipy PAA in the three tissue regions was deduced by subtraction of the values of DM-Bodipy PAA labeling plus (–)bepridil post-treatment from the values of DM-Bodipy PAA labeling alone. Data represent the mean with SE of 80 sections

In one-month-old plantlets, the outermost cell layers of the shoot (including the apex, leaves, and stem) exhibited no fluorescence (data not shown). In contrast, in the root tips, the root cap, the protoderm without root hairs, and the epidermis with root hairs were strongly labeled by DM-Bodipy PAA (Fig. 5a–d). The cell layers covered by the root cap also showed a strong fluorescence signal (Fig. 5c).

In vitro cultured zygotic embryos

In immature zygotic embryos cultured in vitro for 8 days, the lower cotyledon, which was in direct contact to the culture medium, lost its smooth epidermis, enlarged and became callogenic (Fig. 6). In that case, the embryo axis and the upper cotyledon kept the normal developmental pattern of germination (Fig. 6) and still exhibited DM-Bodipy PAA labeling at their outer cell layers. The cells at the periphery of the callus were not labeled by DM-Bodipy PAA.

Callus and somatic embryos

In epidermal strips obtained from seedling hypocotyls and cultured in vitro, the original epidermis, which could still be attached to the callogenic subepidermal tissue, showed a strong labeling (Fig. 7a). Most callus cells exhibited no or only a very weak fluorescence, except in the areas where somatic embryos were to be formed (Fig. 7a). Such somatic embryos developed in the upper cell layers of the explant (Fig. 7b). Calli which did not develop protoderm and epidermis did not show DM-Bodipy PAA labeling. When somatic embryos developed further, they showed a fluorescence pattern which was similar to that of zygotic embryos: DM-Bodipy PAA labeled only the outermost cell layers (Fig. 8).

Discussion

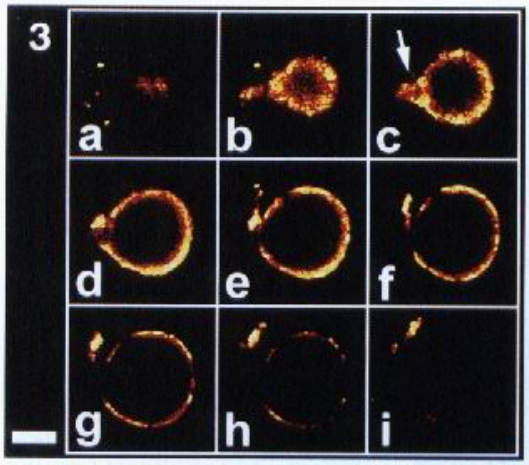
Fluorescent probes provide useful tools to study cytomorphogenesis during embryo development in vivo and in vitro (XuHan et al. 1995, Timmers et al. 1996). DM-Bodipy PAA, a fluorescent probe of the phenylalkylamine type known to label Ca^{2+}-channels of animal cell membranes (Knaus et al. 1992), has been shown to label sunflower cells (Vallée et al. 1997). Because there is a lack of data about the molecular structure of plant Ca^{2+}-channels and the putative specificities of PAA to both Ca^{2+}- and K^+-channels, it is impossible to make a firm statement about the location of Ca^{2+}-channels in sunflower tissues. However, considering that in tested plant materials, PAA binds to plasma membranes (Vallée et al. 1997), inhibits Ca^{2+} influx or blocks Ca^{2+}-channel activity (Piñeros and Tester 1997), binds to isolated Ca^{2+}-channel peptides, and affects the activity of K^+-selective channels (Terry et al. 1992), one may speculate that the location of PAA binding sites in plant tissues is highly related to the distribution of K^+- and especially Ca^{2+}-channels.

In this work we have shown that DM-Bodipy PAA labels embryonic and nonembryonic tissues of sunflower differentially. The labeling of embryo sections and the comparison of fluorescence intensities measured in the protoderm region, near the cut face, and within the inner tissue proved that the intensive labeling of the outer embryonic cell layer was not caused by a limited penetration of the probe into deeper regions of the samples but corresponded actually to a different labeling pattern in the outer and inner cell layers. As the DM-Bodipy PAA labeling in embryonic tissues was strongly reduced by the calcium channel antagonist (–)bepridil, which binds to the same sites as PAA, it is demonstrated that DM-Bodipy PAA binds to specific PAA sites in sunflower tissues.

As summarised in Fig. 9, 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 nonembryonic 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 epidermises.

The strong labeling of the suspensor of the zygotic embryo of sunflower can be related to the critical role to be 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 hypothetical signalling molecules from the endosperm or maternal tissues to embryo proper (XuHan and van Lammeren 1997).

An interesting difference between the labeling of the root and the shoot apex was also observed. In the root, the cell layer underneath the root cap shows a stronger labeling than the cells in the very root cap and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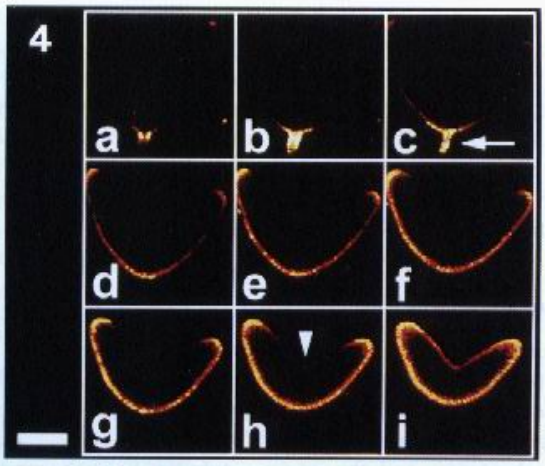
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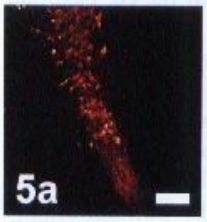
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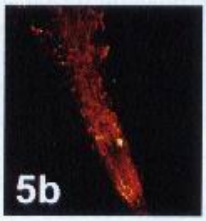
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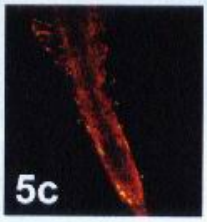
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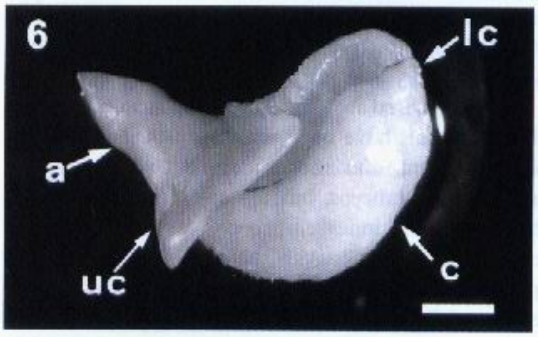
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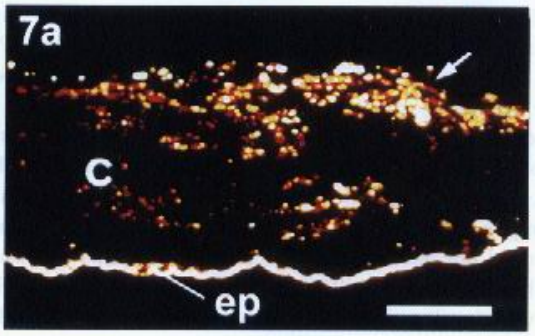
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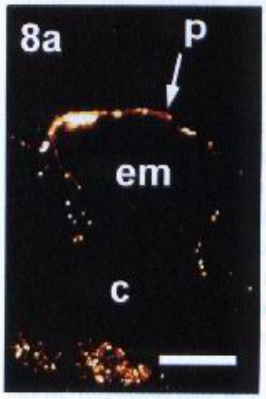
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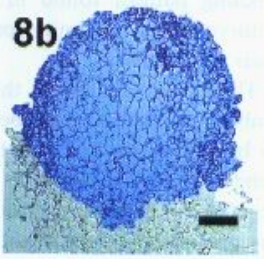
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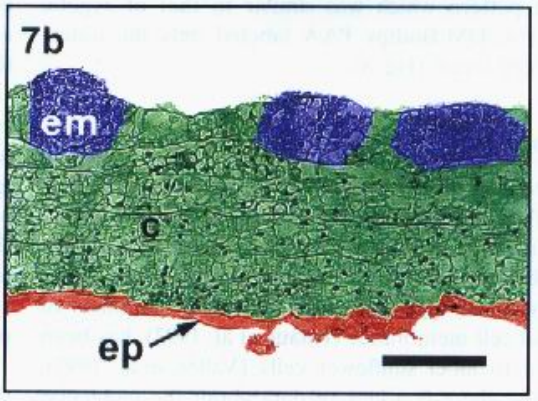
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the inner cells of the root. The root epidermis is also intensively labeled. In the shoot apex the labeling of the outermost cell layers is lower than that in the root. A decrease of the labeling intensity was observed in the shoot apex during germination. Although the epidermises of root and shoot have the same origin: the embryo proper, the root develops from the basal domain and the hypophyse, while the shoot develops from the apical domain (Laux and Jürgens 1997). Thus two hypotheses can be made to explain the observed difference of DM-Bodipy PAA labeling in the epidermal tissues of shoot and root: one is positional and based on the difference of position in the original tissue; the other is functional, involving nutrient transport which could necessitate a higher density of ion channels in the root epidermis. Ion fluxes in plant roots are known to involve Ca^{2+}- and K^{+}-channels (Maathuis et al. 1997, Shabala et al. 1997).

By using DM-Bodipy PAA we were able to distinguish two types of epidermises, one not labeled and

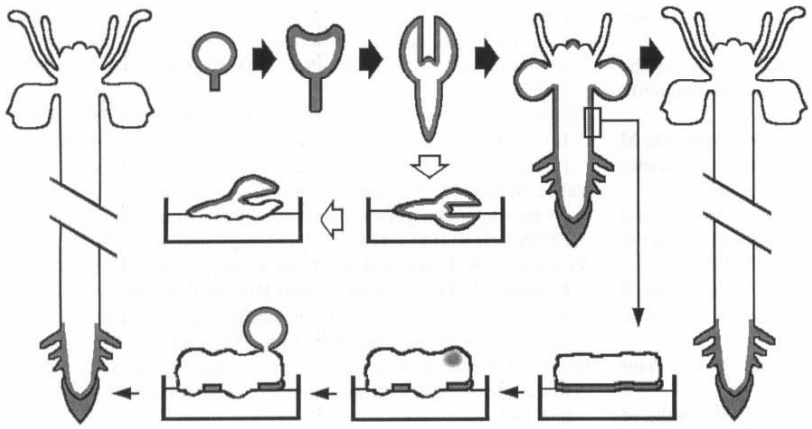

Fig. 9. Schematic representation of DM-Bodipy PAA labeling (grey shades) in embryos and plants of sunflower during the development of zygotic embryos to plants (thick solid arrows), during the culture of zygotic embryos (thick open arrows), and during the culture of hypocotyl strips giving rise to somatic embryos (thin arrows)

Fig. 3a–i. Series of optical sections of an intact zygotic embryo of sunflower 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). There is less or even no fluorescence in distant optical sections (lower row of the series of micrographs) due to reduced transmission of laser light through the embryo proper. Bar: 100 µm

Fig. 4a–i. Series of optical sections of an intact zygotic embryo of sunflower 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. There is no fluorescence in distant serial sections (upper rows of the micrographs) and the shoot apex region (arrowhead) due to reduced transmission of laser light through the thick embryo proper. Bar: 200 µm

Fig. 5. Series of optical sections of an intact root tip of a zygotic embryo of sunflower labeled with DM-Bodipy PAA as observed by CLSM from the outer region (**a** and **b**) towards the median sections (**c–e**). Note the fluorescence in root hairs (**a–e**), epidermis (**c–e**), protoderm (**c** and **d**), and root cap (**c** and **d**). Bar: 1 mm

Fig. 6. Immature zygotic embryo in culture for 8 days. The lower cotyledon (*lc*), which is in contact with the culture medium, developed callus (*c*); the embryo axis (*a*) and the upper cotyledon (*uc*) developed normally. Bar: 1 mm

Fig. 7. **a** Confocal optical section of an epidermal strip in culture, labeled with DM-Bodipy PAA. The original epidermis (*ep*) is strongly fluorescent. Whereas the inner cells of the callus are often only weakly labeled, the cells of the outermost cell layers of the callus, where somatic embryos are formed, are labeled intensively (arrow). **b** Thin section of an epidermal strip in culture, showing the initiation sites of somatic embryos (*em*, blue) within the outermost cell layer of the callus. The original hypocotyl epidermis (red) was still attached to the subepidermal cell layers that became callogenic (*c*, green). Bar: 100 µm

Fig. 8. Somatic embryos of sunflower as observed by CLSM (**a**) and light microscopy (**b** and **c**). **a** Optical section of a somatic embryo of sunflower developed from an epidermal strip in culture. Note the intensive DM-Bodipy PAA labelling of the embryonic protoderm. *em* Embryo, *p* protoderm, *c* callus. Bar: 100 µm. **b** Thin section of a somatic embryo at the globular stage (blue) surrounded by callus cells (green) at its base. Bar: 100 µm. **c** Thin section of a part of a somatic embryo at the heart-shaped stage, showing regularly aligned protoderm cells (red) covering the embryonic ground tissue (green). Bar: 20 µm

functioning in protection, the other intensively labeled and probably also functioning in interaction with the environment by the exchange of ions.

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