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An immunofluorescence study on calmodulin distribution during somatic and zygotic embryogenesis of carrot (*Daucus carota* L.)

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SUMMARY

The distribution of calmodulin was studied during somatic and zygotic embryogenesis and during germination of carrot. Calmodulin levels were visualized by immunofluorescence, using semi-thin sections of different developmental stages of embryogenesis. In proembryogenic masses the distribution of calmodulin was highly variable between individual cells. In later stages of somatic embryogenesis, calmodulin was mainly found associated with organelles in the protoderm. In contrast, in zygotic embryos calmodulin was found to be evenly distributed over the entire embryo. A polarized distribution of calmodulin was observed during germination of the zygotic embryos. Calmodulin was mainly found in the cytoplasm of the epidermis of the cotyledons and the hypocotyl, but was clearly associated with organelles in the root side of the germling. It is concluded that the distribution of calmodulin differs in somatic and zygotic embryos of carrot and that the distribution in somatic embryos resembles the distribution in germinating zygotic embryos.

Key-words: calmodulin, *Daucus carota*, embryogenesis, immunofluorescence.

INTRODUCTION

The whole sequence of plant development, from one cell, the zygote, to the germling, takes place during plant embryogenesis. In nearly all angiosperms, the zygote divides transversally into a basal and an apical cell, after which subsequent cell divisions and cell growth lead to the development of a globular-shaped embryo, demonstrating radial symmetry. In dicotyledons, bilateral symmetry is introduced by flattening of the embryo and the emergence of cotyledons. The embryo in this stage is described as heart-shaped. After elongation of the cotyledons and the then discernible hypocotyl the embryo reaches the torpedo-shaped stage, showing differentiation of apical meristems, procambium and radicle (see, e.g. Esau 1977). In our definition embryogenesis is completed at the stage of the germling and is then proceeded by embryo germination.

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Zygotic embryos can be obtained from flowers, which are very abundant in umbels of carrot plants. However, studies on zygotic embryos are hampered by the presence of surrounding maternal tissue. Therefore, since the discovery of *in vitro* embryogenesis in cultures of *Daucus carota* (Reinert 1958; Steward *et al.* 1958) somatic embryos of carrot are often used as experimental substitutes for zygotic embryos. Carrot somatic embryos can be obtained, essentially free of surrounding tissue, by transferring undifferentiated cell clusters from medium supplemented with the growth regulator 2,4-D to medium without 2,4-D. Embryogenesis can be controlled experimentally to achieve synchronized development and uniform embryogenic stages can be isolated in large amounts (Giuliano *et al.* 1983). These features of carrot make this plant an ideal model system for the study of plant embryogenesis (see also Choi & Sung 1989).

The use of somatic embryos instead of zygotic embryos for experimental purposes assumes a similar developmental pathway. Comparative studies, however, are scarce (see, e.g. Xu & Bewley 1992). For carrot, differences between somatic and zygotic embryos of carrot have been described by Halperin (1966). He states that somatic embryos lack a clearly defined protoderm, may exhibit vascular differentiation earlier than zygotic embryos and have shorter cotyledons. Mature embryos *in vitro* also lack a period of dormancy unlike their counterparts *in ovulo* (Wetherell 1978). These differences, however, do not prevent somatic embryos from growing into normal, full-grown plantlets.

Increasing evidence is available that the divalent cation Ca^{2+} participates in the initiation and maintenance of many plant processes from which several are also important during embryogenesis (e.g. Hepler 1988; Timmers 1990). Of special interest are the regulation of cell polarity, cell division, cell growth, cell volume, plant hormone action and distribution, and enzyme synthesis and activation (Timmers 1990). A key regulatory protein in a number of Ca^{2+}-linked processes is calmodulin (Roberts & Harmon 1992).

In an earlier report we described that calmodulin, visualized by fluphenazine fluorescence, is irregularly distributed in proembryogenic masses of carrot, but that an obvious polar distribution is present in later embryogenic stages (Timmers *et al.* 1989). It is important to know whether this polar distribution holds also true for *in vivo* zygotic embryo development. Use of fluphenazine, however, requires isolation of embryos which could lead to damage and subsequent change in calmodulin distribution. Therefore, study of *in situ* distribution needs other methods, such as histochemistry. This paper describes results obtained by the use of antibodies directed against pea or bovine calmodulin to compare the distribution of this protein during somatic and zygotic embryogenesis of carrot.

Calmodulin has been reported to be present in high amounts during germination of a number of plant species (e.g. Cocucci & Negrini 1991) and comparison of the distribution during zygotic embryo germination with the distribution during somatic embryogenesis may give insight into the mechanism of seed dormancy. Therefore, we also examined the distribution of calmodulin during zygotic embryo germination.

MATERIALS AND METHODS

Plant material and culture conditions

The carrot (*Daucus carota* L.) cultivar Trophy was used. Embryo cultures were maintained in liquid B5 medium (Gamborg *et al.* 1968) with 2 μM 2,4-D, and initiation

of embryogenesis was as described previously (De Vries *et al.* 1988) by transferring the fraction of 50 μm to 125 μm, which contains many proembryogenic masses, to 2,4-D free B5 medium. Flowers of carrot at different stages of development were kindly provided by Zaadunie B.V. (Enkhuizen, The Netherlands). To obtain germinated zygotic embryos, mature seeds were placed in Petri dishes, on filter paper wetted with tapwater, and incubated in the dark at 25°C. Seeds germinated after 2–3 days.

Specimen preparation for immunolabelling

Cell aggregates and somatic embryos were harvested directly after initiation of embryogenesis and during subsequent stages of embryo development. Immature mericarps of carrot were sampled at a size of approximately 3–6 mm, divided into two parts by a tangential cut and de-aerated to improve uptake of the fixative. Germinated zygotic embryos were harvested 3 days after the induction of germination, separated from the mericarp and put into the fixative.

Originally, specimens were fixed in 3% paraformaldehyde in culture medium for 1 hour and rinsed in phosphate buffered saline (PBS: 135 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7·4). Later, another fixation protocol was employed: it was described as optimal for the localization of calmodulin in plant cells and used 3·7% paraformaldehyde in 50 mM phosphate buffer at pH 10 (Wick & Duniec 1986). The specimens were either dehydrated with ethanol (30%, 50%, 70%, 90%, each step taking 10 min, and 3 times 10 minutes 100%) and embedded in polyethyleneglycol (PEG: mixture of 1500 and 4000, 3:2) or stepwise infiltrated to a final concentration of 2·3 M sucrose in PBS (0·1 M, 4 hours; 1·0 M, overnight; 2·3 M, 4 hours), quickly frozen in liquid propane and stored under liquid nitrogen.

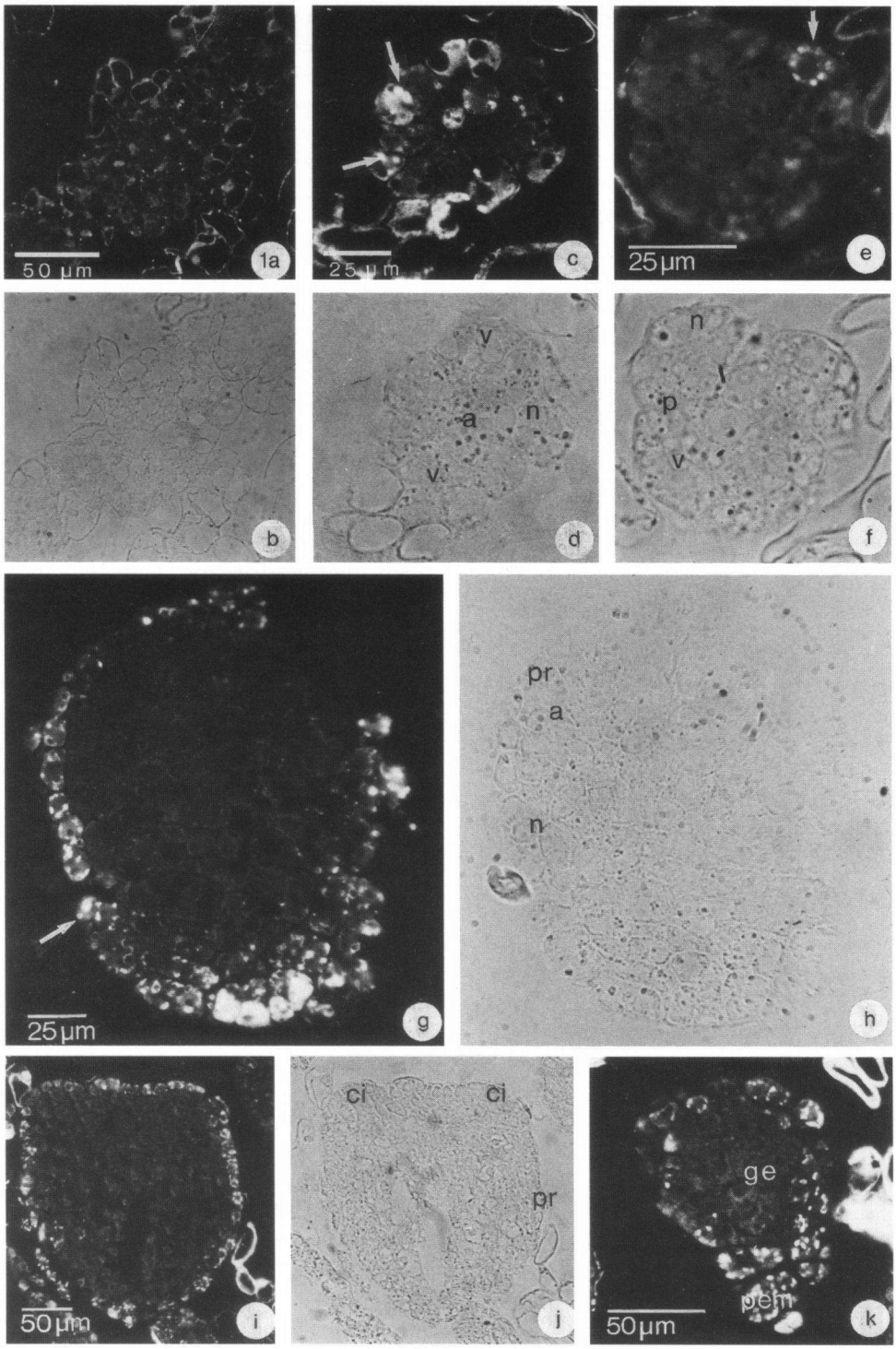
Semi-thin (3–5 μm) PEG sections were made with a Leitz Wetzlar microtome equipped with a steel knife. Ribbons were broken into small pieces, picked up with a drop of 40% PEG 6000 and tipped onto poly-L-lysine (Sigma, St. Louis, M 70 000, 0·1% w/v in distilled water) coated slides. Semi-thin (2–5 μm) cryosections were made with a Reichert Ultracut E ultramicrotome with the FC 4D chamber at −80°C, specimen temperature at −20°C and knife temperature at −35°C. Sections were picked up with a drop of 2·3 M sucrose in PBS, thawed and tipped onto poly-L-lysine coated slides.

Antisera

We used a commercial polyclonal antiserum directed against bovine calmodulin (Calbiochem, San Diego) and a monoclonal antibody (designated FF7) directed against pea calmodulin (a generous gift of P. Jablonsky and R. Williamson, Canberra). The specificity and properties of this antibody are described by Jablonsky *et al.* (1991). The fluorochrome conjugated antisera were respectively rabbit-anti-sheep-FITC and rabbit-anti-mouse-IgM-FITC (Nordic, Tilburg).

Immunolabelling and immunofluorescence

Slides, covered with sections, were rinsed in PBS to remove PEG or sucrose, incubated for 5 min in 0·1 M NH_4Cl, rinsed again in PBS and incubated either with the polyclonal antiserum, diluted 50 or 100 times, or with the monoclonal antibodies, diluted 5 times. After rinsing in PBS the slides were incubated with the appropriate FITC-conjugated antiserum and rinsed again in PBS. All incubations were done at room temperature for 45 min. Finally, sections were embedded in saturated Mowiol 4-88 in Citifluor (Citifluor Ltd, London, UK).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As controls, for non-specific labelling of respectively the second and the first antibody, sections were incubated either with only the second antiserum or with normal mouse serum instead of the anti-calmodulin antibody. Also as a control, sections of mericarps were incubated in antibody treated with an excess of calmodulin isolated from spinach (Sigma, St. Louis, USA).

Observations were made with a Nikon Labophot, using Nikon Fluor 20 × /0·75, Leitz Fluor 50 × /1·00 water and Nikon Plan 100 × /1·25 oil objectives, or a Nikon Microphot-FXA, using Nikon Fluor 20 × /0·75, Fluor 40 × /0·85, PlanApo 60 × /1·40 oil and PlanApo 100 × /1·40 oil objectives, with automatic exposure using the Nikon UFX system. Data were recorded on Kodak Ektachrome P800/1600.

RESULTS

Calmodulin distribution during somatic embryogenesis

The development of carrot somatic embryos from proembryogenic masses to torpedo-shaped embryos was characterized by a conspicuous distribution of calmodulin. In 2,4-D-containing medium the proembryogenic masses usually had a weak and uniform signal (Fig. 1a, b), although in some cases differences in the intensity of labelling of the cytoplasm were found while the fluorescence also could be associated with organelles (Fig. 1c, d).

In 2,4-D-free medium the overall image of the distribution of the signal was more consistent. Shortly after the initiation of embryogenesis, proembryogenic masses with only one cell (Fig. 1e, 1f, arrow) or a few cells with an organelle associated fluorescence were observed. From the globular to the torpedo-shaped embryo, calmodulin was most obviously present in the outer layer, the protoderm, of the embryo (Fig. 1g–k). It was typical that, at this stage also, the cells of the proembryogenic mass showed the organelle associated fluorescence (Fig. 1k).

The fixation at pH 7 gave acceptable results, but the intensity of the signal was improved by fixation at pH 10. Consequently, the protocol with high pH fixation was used and all figures presented here are of material treated this way. Cryosections were, in most cases, superior to sections of PEG embedded material if one compared the general preservation of cellular structure. Both embedding methods, however, showed no difference in distribution nor intensity of the fluorescent signal. No striking differences were observed after immunolabelling with the polyclonal antiserum, directed

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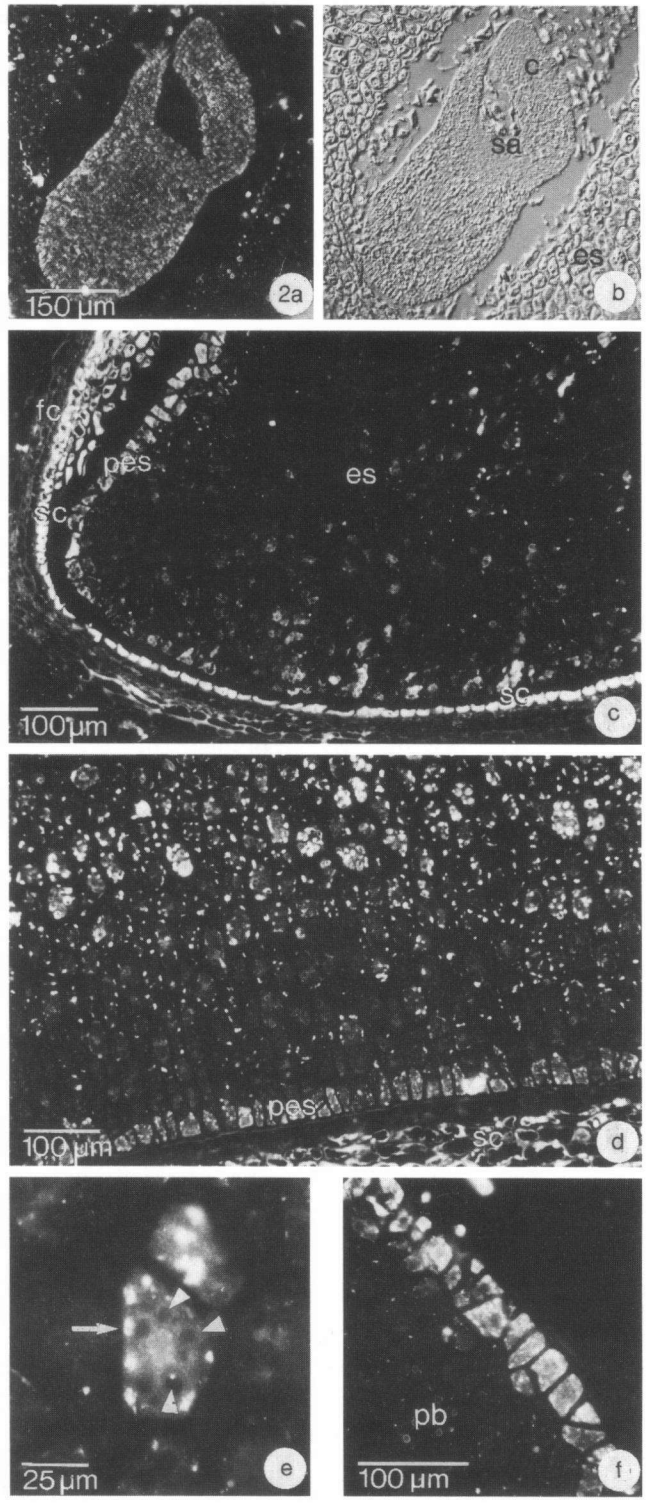
Fig. 1. Distribution of calmodulin in sections of a carrot suspension culture during somatic embryogenesis. (a) Cryosection of a proembryogenic mass. The fluorescence has a low intensity and is distributed uniformly over all cells. (b) The bright field of image of (a). (c) Another cryosection of a proembryogenic mass. Differences in fluorescence intensity occur in some cells. The signal is organelle associated (arrows). (d) The bright field image of (c). (e) Cryosection of a proembryogenic mass 1 day after initiation of embryogenesis. Only one cell shows an organelle associated fluorescence (arrow), while the others are only weakly and uniformly labelled. (f) The bright field image of (e). (g) A globular somatic embryo showing the typical distribution of calmodulin in somatic embryos. The highest fluorescence in this cryosection is present in organelles surrounding the nucleus of protoderm cells (arrow). At the base of the embryo 2–3 cell layers contain the signal. (h) The bright field of image of (g). (i) Cryosection of a heart-shaped somatic embryo with calmodulin labelling present in all cells of the embryo. (j) The bright field image of (i). (k) Cryosection of a globular somatic embryo with the proembryogenic mass still attached to it. At this stage also these cells show fluorescent organelles. a, amyloplast; ci, cotyledon; ge, globular embryo; n, nucleus; p, plastid; pem, proembryogenic mass; pr, protoderm; v, vacuole. All sections were labelled with the monoclonal FF7 antibody.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against bovine calmodulin, or with the monoclonal antibody, directed against pea calmodulin.

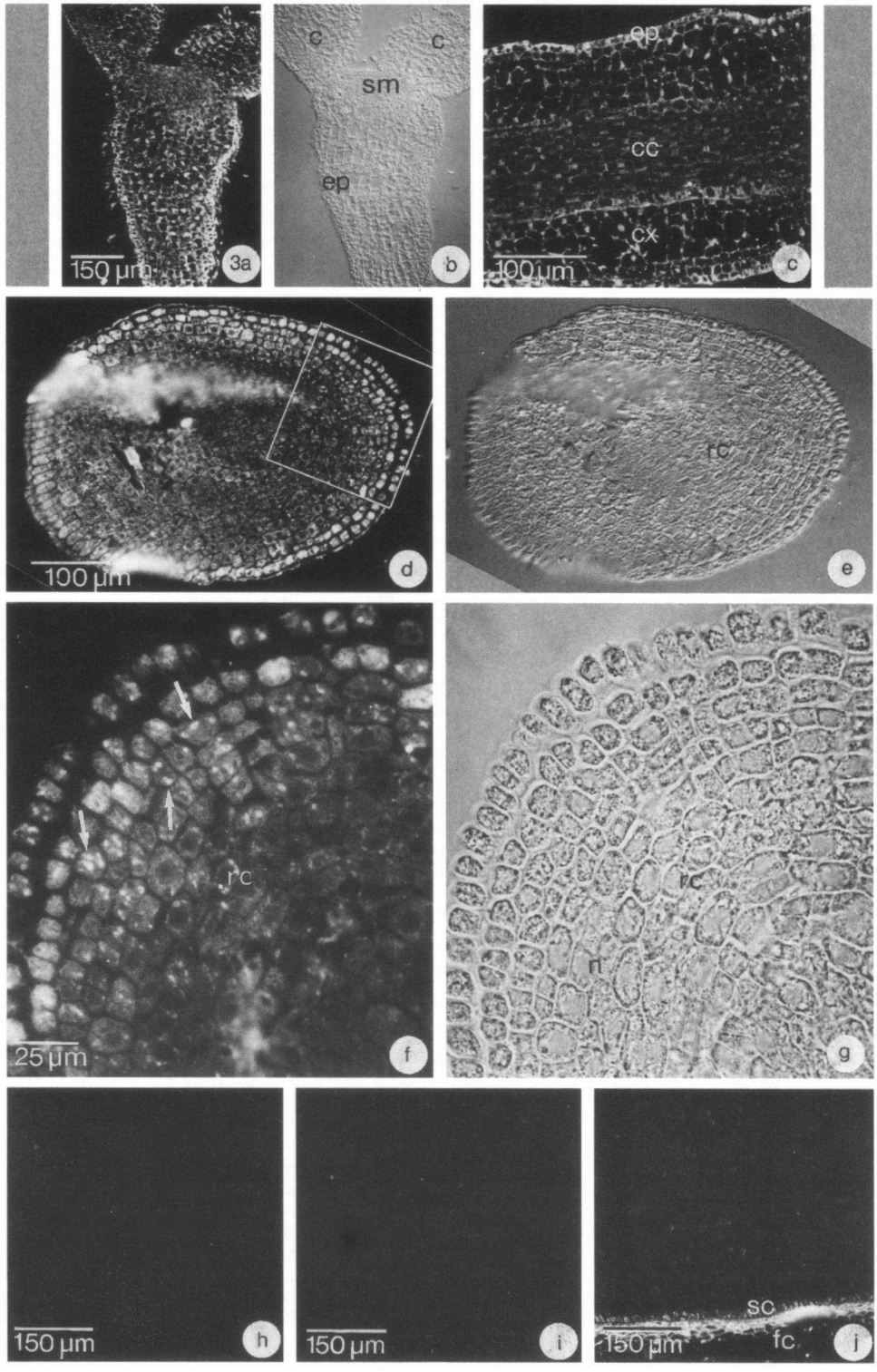
In control sections proembryogenic masses and embryos were usually negative, although sometimes a weak signal was present. After using only the second antiserum no signal was observed in proembryogenic masses or embryos (results not shown). Replacing the first antibody with normal mouse serum resulted in embryos with a granular fluorescence in vacuoles of the future cortex of the embryo, among a weak signal from the other cells. The large vacuolated cells showed an organelle associated or cytoplasmic fluorescence in both controls. The typical organelle associated fluorescence of the protoderm, characteristic for somatic embryos, was never observed in control sections.

Calmodulin distribution in immature seeds

The distribution of calmodulin was observed in mericarps of about 3–6 mm with a well developed cellular endosperm and an embryo of approximately 400–1000 μm in length. In the zygotic embryo, the fluorescent signal was distributed uniformly over the embryo with no obvious differences between the protoderm and other cell layers of the embryo (Fig. 2a, b). Higher magnifications revealed a punctate labelling in the cytoplasm, surrounding the nucleus or scattered over the cytoplasm. Nuclei were negative or slightly positive with a negative nucleolus. In contrast with the uniform distribution of the signal in the embryo, the distribution in the endosperm was eminently variable. The outermost cell layer in the mid region of the endosperm, in cereals often described as the aleurone layer, showed a strong fluorescence in comparison with the other cells of the endosperm. Five to six cells layers further inside the endosperm, almost without any signal, were followed by cells with a conspicuous, punctate fluorescence (Fig. 2d). In most cases the strong fluorescence of the outermost layer was restricted to one side of the endosperm. Another strongly fluorescent layer, directly adjacent to the endosperm, could be observed (Fig. 2c). This layer, the immature seed coat, forms the outer layer of the integuments between the two linked mericarps, but lies directly against the endosperm at the opposite side. Also the remnants of the integuments were strongly fluorescent.

At higher magnifications the differences between the various tissues became more clear. The strong signal of the outermost endosperm layer was localized in the cytoplasm. The nuclei were only faintly fluorescent, while nucleoli were negative. The peripheral, punctate fluorescence was also found in these cells (Fig. 2e). In the centre of the endosperm the signal was most obvious in particles at the cell edge (Fig. 2f, arrow). Occasionally, cells with the punctate fluorescence were found as a group, 2–3 cell layers wide and 8–9 cells long, oriented from the edge of the endosperm to the centre of it. At

Fig. 2. Distribution of calmodulin in immature seeds of carrot. (a) Cryosection of a torpedo-shaped zygotic embryo showing the typical distribution of calmodulin in zygotic embryos. No obvious differences are visible between different cell layers. (b) The DIC image of (a). (c) A longitudinal PEG section through the top of an immature seed. Note the presence of the highly fluorescent fruit coat directly adjacent to the endosperm at the convex side of the seed. (d) A longitudinal PEG section through the middle of the concave side of an immature seed. The seed coat and peripheral endosperm are strongly labelled. More inside the endosperm, a conspicuous punctate labelling can be observed. (e) Higher magnification of a cell in the middle of the endosperm. The label is concentrated in particles at the cell edge (arrow); protein bodies are negative (arrowheads). PEG embedding. (f) Detail of the outer cell layer of the endosperm; the label is present in the cytoplasm, but also a punctate peripheral localization can be seen. PEG embedding. c, cotyledon; es, endosperm; fc, fruit coat; pb, protein body; pes, peripheral endosperm; sa, shoot apex; sc, seed coat. All sections labelled with the FF7 antibody.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the micropylar region the strong fluorescence of the endosperm was present in 2–3 cell layers. If present, large vacuoles were always negative. The strong fluorescence of the immature seed coat had a threadlike appearance and was almost uniform in intensity.

Calmodulin distribution in germinated zygotic embryos

The distribution of calmodulin in germinated zygotic embryos was strikingly different from the distribution during zygotic embryo development. From cotyledons to root tip most of the signal was present in the epidermis, especially in the region below the cotyledons (Fig. 3a, b). The shoot meristem did not show any noticeable fluorescence. In the cortex of the hypocotyl many nuclei were positive and a scattered punctate fluorescence was observed in these cells. Besides a stronger fluorescence of the epidermis no obvious signal was present in median sections of germinated embryos (Fig. 3c). The fluorescence of the epidermis was mainly localized in the cytoplasm.

The distribution of the signal in root sections through germinated embryos, with a root length of a few millimetres already, was very characteristic. Two to three peripheral cell layers were strongly fluorescent and the 3–4 adjacent cell layers showed an organelle associated fluorescence (Fig. 3d,e). The innermost cells were not conspicuous in fluorescence. At a higher magnification it was observed that most peripheral cells possessed 3–4 organelles with a strong signal (Fig. 3f, arrows). From their appearance and location these organelles were judged to be proplastids or small amyloplasts.

Controls

Control sections were always much weaker in intensity than sections treated with anti-calmodulin serum. Sections treated with only the second antibody (Fig. 3a) or treated with normal mouse serum instead of the anti-calmodulin serum (Fig. 3b) were totally negative. Sections treated with anti-calmodulin antibody pretreated with an excess of spinach calmodulin (Fig. 3c) showed a very reduced signal. Since in the latter case a signal was always present in a part of the pericarp of the immature seed (Fig. 3c), we take this for non-specific labelling.

DISCUSSION

The overall distribution of calmodulin in somatic embryos of carrot differs strikingly from the overall distribution in zygotic embryos. Immunolocalization of calmodulin revealed a high concentration of calmodulin located in the protoderm of somatic embryos. In zygotic embryos calmodulin was found to be evenly distributed over all cell layers. In germinated zygotic embryos, however, a specific localization of calmodulin was again observed with a higher amount of calmodulin in the peripheral layers of the

Fig. 3. (a)–(g). Distribution of calmodulin in sections of germinated zygotic embryos of carrot. (a) Median longitudinal cryosection through a seedling showing the distribution of calmodulin in the cotyledons and hypocotyl. The highest signal is present in the epidermis. (b) The DIC image of a. (c) A higher magnification of the hypocotyl of a germinated embryo. (d) Oblique cross-section through the root tip. Note the organelle associated fluorescence of the peripheral cell layers. (e) The DIC image of d. (f) Higher magnification of the area outlined in the box in d. Most of the signal is present in amyloplasts of the peripheral cell layers (arrows). (g) The bright field image of f. (h)–(j). Control cryosections. They were made from the convex side of an immature seed of carrot. (h) First antibody replaced with PBS. (i) First antibody replaced with normal mouse serum. (j) First antibody incubated with an excess of calmodulin from spinach before applying to the section. c, cotyledon; cc, central cylinder; cx, cortex; ep, epidermis; fc, fruit coat; n, nucleus; rc, root cortex; sc, seed coat; sm, shoot meristem. All sections were labelled with the FF7 antibody.

young seedling. At the cellular level, calmodulin was found predominantly in plastids in somatic embryos. In zygotic embryos, calmodulin was present in the cytoplasm at a low, evenly distributed, level with higher amounts in small organelles, which were found scattered over the cytoplasm. In germinated zygotic embryos, calmodulin was present in the highest amount in amyloplasts in the peripheral layers of root sections.

All reports on calmodulin localization after tissue destruction describe a predominantly cytoplasmic localization of calmodulin. Here, and in other immunofluorescence studies, an obvious organelle associated localization was uncovered. Butcher & Evans (1986) found calmodulin localized in protein body like organelles surrounding the nucleus in pea roots. Dauwalder *et al.* (1986) described a vacuolar presence of calmodulin and a general association of calmodulin with plastids. Recently, Overvoorde & Grimes (1994) reported a spot-like distribution of calmodulin in carrot somatic embryos. An explanation for this difference in calmodulin localization could be the presence of a loosely bound or easily extractable calmodulin in these organelles, which could be lost during tissue destruction and organelle isolation and would increase the cytoplasmic portion (see also Van Eldik & Watterson 1985; Andreev *et al.* 1990). On the other hand, it is clear that during fixation and processing of tissue for immunolabelling, proteins can be redistributed or differentially be extracted (Melan & Sluder 1992). A specific organellar localization might be the result of redistribution of cytoplasmic calmodulin during fixation of specific loss of calmodulin from the cytoplasm. Redistribution over different cell-layers, however, must be excluded.

In spite of the above-mentioned drawbacks, we think it feasible to state that the amount and distribution of calmodulin vary strongly between different cells of one organism. Therefore, in addition to switching from the Ca^{2+} free to the Ca^{2+} bound form, the action of calmodulin can directly be controlled by its level and distribution (see also Allan & Hepler 1989; Trewavas 1991) in contrast with the opinion of others (see the review by Poovaiah 1985) who state that the amount of calmodulin is never a limiting factor for its action. A tissue-specific difference in calmodulin amount was also described for pea seedlings by Allan & Trewavas (1986), for a number of plant species by Lin *et al.* (1986), and for maize seedlings by Stinemetz *et al.* (1987). They all found that calmodulin is present in the highest amount in root tips. According to Poovaiah *et al.* (1987) root tips contain up to four times more calmodulin compared with the root base.

The presence of calmodulin in plastids implies a role for calmodulin in starch biosynthesis and degradation during plant growth and differentiation (Mitsui *et al.* 1984; Preusser *et al.* 1988; Dreier *et al.* 1992), possibly by reversible, Ca^{2+}-dependent, phosphorylation of proteins as was described for amyloplasts of sycamore cells (Ranjeva & Boudet 1987). In *Nicotiana tabacum* and *Datura innoxia* cell cultures, a protein kinase was isolated from plastids and the activity of this enzyme correlates with rapid cell proliferation and starch accumulation (Böcher *et al.* 1985). Embryogenesis and germination coincided with changes in the amount of starch and plastid differentiation and development (Halperin & Jensen 1967; Tisserat *et al.* 1979; Wurtele *et al.* 1988). Plastids are, together with vacuoles, the most variable structures during morphogenesis (Buvat 1989). Therefore, a first step in somatic embryogenesis might be a change in starch metabolism modulated by calmodulin. Michaux-Ferriere *et al.* (1992) described a change in starch composition of plastids during somatic embryogenesis of *Hevea brasiliensis.* A change in the amount of calmodulin during germination is described by Cocucci & Negrini (1991) for *Phacelia tanacetifolia* seeds. These authors

linked calmodulin increase with the metabolic reactivation during germination. Another possibility is considered by Dauwalder *et al.* (1986) who related the presence of calmodulin in plastids, in the columella cells of the root cap, with signalling of changes in root orientation (see also Poovaiah *et al.* 1987).

A preferential presence in the protoderm during somatic embryogenesis is not restricted to calmodulin. Kiyosue *et al.* (1992) describe the presence of an embryogenic protein of carrot which appears in the protoderm of torpedo-shaped embryos after treatment with ABA. Sterk *et al.* (1991) found an almost exclusively protodermal presence of mRNA coding for a lipid transfer protein during carrot embryogenesis. Also, concanavalin A-stainable glycoproteins are restricted to the protoderm in somatic embryos of carrot (Lo Schiavo *et al.* 1990). These facts point to the importance of the development of the protoderm during embryogenesis, as was also stated by Bruck & Walker (1985), Mayer *et al.* (1991) and De Jong *et al.* (1992) and is in strong contradiction to the statement of Halperin (1966) that somatic embryos do not develop a defined protoderm.

In immature seeds calmodulin was conspicuously present in the outer layer of the endosperm. GP80, a glycoprotein isolated from carrot fruits, was also found predominantly in this layer (Torii *et al.* 1991). In Graminales the outer layer of the endosperm forms the aleurone layer, which is responsible for the hydrolysis of carbohydrates stored in the inner 'starchy' endosperm at germination (for review, see Jacobsen 1984). Ca^{2+} is involved in the regulation of the synthesis and activity of α-amylase in the barley aleurone (e.g. Bush *et al.* 1989). With regard to this the observations of Tretyn & Kopcewicz (1988) on the aleurone layer of oat are also interesting. Using CTC fluorescence and X-ray microanalysis these authors found a high level of Ca^{2+} to be present specifically in this aleurone layer. The distribution of calmodulin in the endosperm might point to a role for calmodulin in starch breakdown during germination of carrot seeds.

Carrot somatic embryogenesis is considered to be a good model system for zygotic embryogenesis (e.g. Choi & Sung 1989). From our results, however, it can be concluded that, at least regarding the distribution of calmodulin, striking differences exist between somatic and zygotic embryos and that strong similarities exist between somatic embryogenesis and zygotic embryo germination. The proper development of the embryo depends on the establishment of polarity during the transition from the globular to the heart-shaped stage. The prevention of this establishment by, e.g. heat shock (Zimmerman *et al.* 1989) or addition of 2,4-D (Sung *et al.* 1984), leads to abnormal embryo growth and giant globular-shaped embryos. It is, therefore, surprising that, during zygotic embryogenesis, in contrast with somatic embryogenesis, no polarized distribution of activated calmodulin, as visualized by fluphenazine fluorescence (Timmers *et al.* 1989), was observed. Yet, zygotic embryos are surrounded by endosperm and connected with maternal tissues which are involved in the proper development of the embryo and the establishment of its polarity. During somatic embryogenesis these tissues are absent and the establishment of polarity has to reside in the embryo itself. A comparable expression of tissue polarity in zygotic embryos, as seen during somatic embryogenesis, was not found until germination. From this the conclusion can be made that, physiologically, somatic embryos resemble germinating zygotic embryos. The absence of dormancy in tissue culture of carrot can then be considered as a direct consequence of this. Somatic embryos develop directly into seedlings, without a period of dormancy, because they have

already started germination at the beginning of their development, i.e. after transfer of proembryogenic masses to auxin-free medium.

The most prominent difference observed between the two types of embryogenesis was the localization of calmodulin in plastids. Plastids, together with vacuoles, are the most variable structures with regards to morphology (Buvat 1989) but, in addition to the differentiating evolution of the cell, they are influenced by many factors, such as the time of the day and, in tissue culture, medium composition. These differences can therefore be the direct consequence of the differences in osmolarity and nutrient composition of the medium surrounding the somatic or zygotic embryo. Somatic embryos generally grow and develop under hypotonic conditions (Fry 1990), while the fluid surrounding the zygotic embryos is considered to be more isotonic with the embryo (Gates & Greenwood 1991). The optimal sucrose concentrations during embryogenesis *in vitro* are reported to decrease with the development of the embryos. More negative osmotic values during early embryogenesis are supposed to slow down growth, thereby permitting normal differentiation and development. Increased levels of sucrose during the culture of mature stages of embryogenesis prevent precocious germination by inhibiting exponential growth of the embryo (Gates & Greenwood 1991).

The structural differences observed between somatic and zygotic embryos can, accordingly, be regarded as resulting from differences in the composition of the medium surrounding the embryo. It remains unclear whether these differences are not only the consequence of exogenous factors, but are also inherent to the processes of somatic and zygotic embryogenesis and reflect a more fundamental difference between the two. However, it is obvious that, if compared to *in vivo* plant development, during carrot somatic embryogenesis two processes intermingle, i.e. embryogenesis and germination. For practical plant breeding this could mean that comparative studies on zygotic embryogenesis, germination and somatic embryogenesis of certain plant species, might lead to more insight into the physiology of somatic embryogenesis.

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