Planta (2002) 215: 577–583
DOI 10.1007/s00425-002-0791-8

ORIGINAL ARTICLE

**Clément Thomas · Roberte Bronner · Jean Molinier
Els Prinsen · Harry van Onckelen · Günther Hahne**

Immuno-cytochemical localization of indole-3-acetic acid during induction of somatic embryogenesis in cultured sunflower embryos

Received: 12 January 2002 / Accepted: 19 March 2002 / Published online: 24 May 2002

Abstract Immature zygotic embryos of sunflower (*Helianthus annuus* L.) produce somatic embryos when cultured on medium supplemented with a cytokinin as the sole source of exogenous growth regulators. The timing of the induction phase and subsequent morphogenic events have been well characterized in previous work. We address here the question of the role of endogenous indole-3-acetic acid (IAA), since auxins are known to have a crucial role in the induction of somatic embryogenesis in many other culture and regeneration systems. The fact that in the sunflower system no exogenous auxin is required for the induction of somatic embryos makes this system very suitable for the study of the internal dynamics of IAA. We used an immunocytochemical approach to visualize IAA distribution within the explants before, during and after the induction phase. IAA accumulated transiently throughout cultured embryos during the induction phase. The detected signal was not uniform but certain tissues, such as the root cap and the root meristem, accumulated IAA in a more pronounced manner. IAA accumulation was not restricted to the reactive zone but the kinetics of endogenous variations strikingly mimic the pulse of IAA that is usually provoked by exogenous IAA application. The direct evidence presented here indicates that an endogenous auxin pulse is indeed among the first signals leading to the induction of somatic embryogenesis.

C. Thomas (✉) · R. Bronner · G. Hahne
Institut de Biologie Moléculaire des Plantes,
CNRS et Université Louis Pasteur,
12 rue du Général Zimmer,
67084 Strasbourg Cedex, France
E-mail: clement.thomas@ibmp-ulp.u-strasbg.fr
Fax: +33-3-88614442

J. Molinier
Friedrich Miescher Institut,
Maulbeerstrasse 66, 4058 Basel, Switzerland

E. Prinsen · H. van Onckelen
Department of Biology,
University of Antwerp (UIA), 2610 Antwerp, Belgium

Keywords *Helianthus* · Immunolocalization · Indole-3-acetic acid · Somatic embryogenesis

Abbreviations BAP: 6-benzylaminopurine · 2,4-D: 2,4-dichlorophenoxyacetic acid · EDAC: 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride · IAA: indole-3-acetic acid · IZE: immature zygotic embryo

Introduction

Somatic embryogenesis is the capacity of somatic or non-sexual plant cells to form embryos. It was shown that the process resembles zygotic embryogenesis (Zimmerman 1993). Since Steward et al. (1958) and Reinert (1958) published the first independent reports of somatic embryogenesis in cultures of carrot cells, somatic embryogenesis has been studied at different levels, such as histological or molecular (Dodeman et al. 1997). A striking common trait in most somatic embryogenesis systems is the requirement for exogenous auxin application (Mordhorst et al. 1997). This makes auxin one of the essential actors in somatic embryogenesis induction. However, once the induction period is accomplished, the process generally continues on medium lacking auxin since a high auxin concentration in the medium is known to inhibit somatic embryo development (Halperin 1995).

The role of exogenous auxin has been largely discussed. However, the role and variations of endogenous auxin are not clearly established. The masking effects of the exogenously supplied auxin, which is taken up from the culture medium, complicate investigations into this subject. In some cases, such as geranium, somatic embryogenesis can be induced on explants cultured on auxin-free medium (Hutchinson et al. 1996). In such situations, induction of somatic embryogenesis is thought to be controlled by the production or redistribution of endogenous auxin.

Immature zygotic embryos (IZEs) of sunflower (*Helianthus annuus* L.) give rise to a well-characterized

embryogenic response (localization and timing) when they are cultured on an auxin-free medium (Bronner et al. 1994). The only growth regulator supplied in this culture system is a cytokinin, 6-benzylaminopurine (BAP). Since no auxin is externally added, we hypothesized that the specific culture conditions lead to a redistribution, production or release from inactive conjugated forms of endogenous auxin within the zygotic embryo. Under this scenario, one would expect a transient and local accumulation of the natural active form of auxin in the morphologically competent zone of the cultured sunflower explants. This hypothesis is impossible to verify by only the use of conventional hormone quantification, e.g. enzyme-linked immunosorbent assay or mass spectrometry, since spatial information is lost during the extraction step. The present investigation addresses the spatial distribution of indole-3-acetic acid (IAA) in cultured IZEs of sunflower, using an immunocytochemical approach, during the period decisive for induction of somatic embryogenesis.

Materials and methods

Plant material

Experiments were carried out using the *Helianthus annuus* L. inbred line HA 300B, kindly provided by Rustica Prograin Génétique (Mondoville, France). Plants were grown under greenhouse conditions and pollinated as previously described (Bronner et al. 1994).

Culture media and tissue culture

Immature zygotic embryos (6 mm long) were harvested 10–12 days after pollination, sterilized, and dissected to obtain longitudinal halves (see Fig. 1a, b). Explants were cultured with the cut surface in contact with the medium according to Bronner et al. (1994). The medium (M12B) contained Murashige and Skoog (1962) macro- and micro-elements, Gamborg B5 vitamins (Gamborg et al. 1968), 1 g l^{-1} casein hydrolysate, 100 mg l^{-1} *myo*-inositol, 500 mg l^{-1} 2-(N-morpholino)ethanesulfonic acid, 0.7 % agar, 12 % sucrose and 6.6 μM BAP. M12 medium was identical but without BAP. Explants were cultured in the dark at 21 °C. Each explant pair was individually identified. First halves were collected at different culture times for further analysis. The second halves were kept in culture to continue their normal development for 2 weeks in order to verify the correct morphogenic response.

Fig. 1a–c. Dissection of explants and induction of somatic embryogenesis in sunflower (*Helianthus annuus*). **a** Freshly excised IZE of 6 mm. *Double-arrowed line* indicates the section produced before culture. **b** Pair of explants obtained after sectioning one IZE. *Bars on the hypocotyl* indicate the morphogenic zone. **c** Somatic embryogenesis induced from the morphogenic zone after 2 weeks of culture on M12B medium. *cot* Cotyledon, *se* somatic embryo. Bars = 1 mm

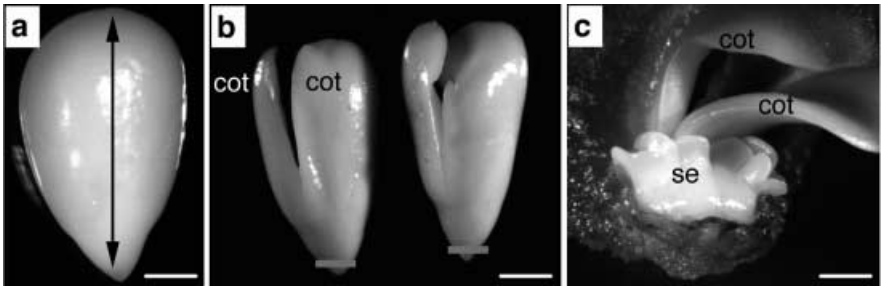
Fixation of samples and preparation of semi-thin sections

The explants were immediately pre-fixed for 1 h at 4 °C, after a brief vacuum treatment, in freshly prepared 4 % aqueous 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDAC; Sigma). The EDAC pre-fixation cross-links IAA to structural proteins within the tissues and preserves the antigenicity of the IAA towards the anti-IAA monoclonal antibody used in this study (Shi et al. 1993; Caruso et al. 1995). Subsequently, the explants were post-fixed for 4 h in FAA (10 % formalin, 5% acetic acid, and 50% ethanol, in water) at 4 °C. The fixed tissues were then dehydrated in an alcohol series, embedded in paraffin, sectioned at 10 μM and submitted to the immunolocalization procedure.

IAA immunolocalization

The anti-IAA monoclonal antibody used was raised against carboxyl-linked IAA in mice (Caruso et al. 1995). It was kindly provided by Dr. J.L. Caruso (University of Cincinnati, Ohio, USA), or bought from AGDIA (Elkhart, Indiana, USA). The procedure for the immunolocalization of IAA has been described in detail by Moctezuma (1999). Briefly, the sections were incubated for 45 min in a blocking solution [10 mM phosphate buffer pH 7 (PBS), 0.1% Tween-20, 1.5% glycine and 5% bovine serum albumin (BSA)] and incubated overnight at 4 °C with the primary antibody (1 mg ml^{-1}) diluted 1:20 in PBS/BSA solution (0.8% BSA). Subsequently, they were incubated for 4 h at room temperature with the secondary antibody [anti-mouse IgG alkaline phosphatase conjugate (1 mg ml^{-1}); Promega, USA] diluted 1:1,000 in PBS/BSA solution. After washing, the sections were developed with 0.34 μg/μl NBT (nitro blue tetrazolium)/0.18 μg/μl BCIP (5-bromo-4-chloro-3-indolylphosphate). As the color developed (15 min) on the sections, they were rinsed with stop buffer (100 mM Tris HCl, pH 8; 1 mM EDTA), dehydrated, mounted (Mounting Medium; Sigma), observed and photographed. All experiments were repeated at least 3 times, starting from independent cultures.

Before proceeding to the immunolocalization of IAA in IZE explants, the effectiveness of the immuno-cytochemical technique and the specificity of the monoclonal IAA antibody were verified on sunflower leaves. Young leaves (< 3 cm) were harvested from 2-week-old plants and either used directly for immuno-cytochemistry or treated as follows. Leaves were depleted of endogenous IAA as described by McClure and Guilfoyle (1987): leaf strips (0.5 cm width) were incubated for 4 h in KPSC medium (2% sucrose, 50 μg/ml chloramphenicol, 10 mM potassium phosphate, pH 6). The incubation medium was changed after 1 h and 2 h. After this incubation period, leaf strips were loaded with auxin by incubation in fresh KPSC medium containing 200 μM IAA or 200 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for 2 h. Control samples were subjected to the same treatment but without added hormone. The samples were then processed as described above.



Hormone extraction and analysis by mass-spectrometry

IZE explants were cultured on M12B medium and sampled at 0, 6, 14, 24 or 48 h. Cotyledons were removed and the resulting embryo axes were immediately frozen in liquid nitrogen. At least 30 axes from 3 independent experiments were collected and pooled for each time point. The combined frozen material was weighed. IAA was extracted overnight from approximately 30 mg of frozen tissue in $CHCl_3$/methanol/water/acetic acid (Bieleski 1964) and purified by solid-phase extraction. The stable isotope [$^{13}C_6$]indole-3-acetic acid (50 ng; Cambridge Isotope Lab, Cambridge, Mass., USA) was initially added as internal tracer. The IAA content of embryo axes was measured using the LC-MS/MS procedure as described previously by Prinsen et al. (1995). The results are expressed as fmol (mg FW)$^{-1}$.

Results and discussion

The embryogenic response in immature sunflower embryos is induced very specifically in a particular region and a direct manner by precisely known culture conditions. The explants are longitudinally sectioned halves of immature embryos, which are cultured with the cut surface turned towards the medium (Fig. 1a, b). The decisive factors triggering the morphogenic response that must be present in the culture medium are a cytokinin, usually BAP, as well as an elevated (12%) concentration of sucrose (Bronner et al. 1994). The induction of the first cell divisions specifically involved in the embryogenic response occurs within a few hours and is limited to a precisely identified morphogenic zone located at the upper border of the hypocotyl-radicle zone (Fig. 1b; Bronner et al. 1994). Somatic embryos are well developed within 2–3 weeks of culture (Fig. 1c).

Quantification of endogenous free auxin

Endogenous free IAA was measured in pooled samples (> 30 embryo axes) originating from three separate experiments. Each datum point thus represents a mean value, reflecting both variations between individual embryos and between experiments. The expected morphological reaction was verified for each embryo since the remaining half was cultured until the response was unequivocally visible. Only embryos with the expected response (80–90 %) were used for quantification. IAA was extracted from samples after 0, 6, 14, 24 and 48 h of culture on M12B and quantified by mass-spectrometry. Freshly isolated immature embryos contained a significant concentration of free IAA [3,970 fmol (mg FW)$^{-1}$], which increased further within the first hours of culture to reach a maximal value of 8,595 fmol (mg FW)$^{-1}$ after 14 h of culture (Fig. 2). This time point is of importance, as the first periclinal divisions, specific for the morphogenic response become visible at this moment (Jeannin et al. 1998). At 24 and 48 h of culture the IAA concentration dropped to values below that of the freshly isolated embryos.

In order to visualize the spatial distribution of IAA within the embryos at the time of induction of the embryogenic response, immuno-cytochemical studies were performed at 14 h, as well as at two time points bracketing this crucial time point, i.e. 0 and 24 h.

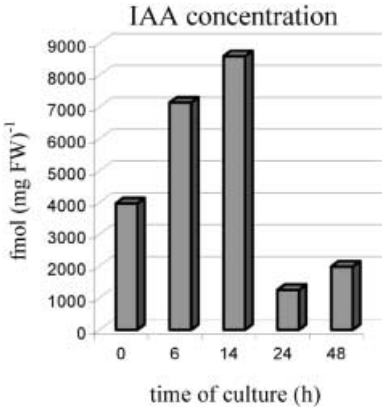


Fig. 2. Free-IAA content in axes of sunflower IZEs after different times of culture on M12B medium. For each time point, IAA was extracted from at least 30 explants originating from 3 independent experiments and quantified by LC-MS/MS. Results are expressed as fmol of IAA per mg of fresh weight (*y*-axis) as a function of time of culture (*x*-axis)

Validation of the immuno-cytochemical approach in sunflower tissues

Immuno-cytochemistry has been successfully employed to localize IAA in different systems, such as peach seedlings (Ohmiya et al. 1990), leaf cells of *Prunus* (Ohmiya and Hayashi 1992), primary roots of corn (Shi et al. 1993) and gynophores of peanut (Moctezuma 1999; Moctezuma and Feldman 1999). However, this approach is delicate and prone to artifacts. The specificity of the reaction of the employed antibody must be validated in the context of the species studied. To this end, we used strips of sunflower leaves because they are easily accessible to the necessary treatments.

Specificity of the immuno-chemical reaction

Transverse sections of young sunflower leaves were used for the validation of the protocol for immuno-cytochemical visualization of IAA. The fixation procedure involved two separate steps. Prior to the usual fixation of the tissue with FAA, IAA must be bound to the tissue by a treatment with EDAC. EDAC cross-links the carboxyl group of IAA to structural proteins while preserving the antigenicity of IAA to this particular monoclonal antibody (Shi et al. 1993; Caruso et al. 1995). The linked IAA can then be visualized by classical immuno-cytochemistry.

A strong signal was detected in young sunflower leaves (Fig. 3a). This observation is in agreement with previous observations that young leaves and apical buds of dicotyledons are rich in IAA (Sitbon et al. 1991). No

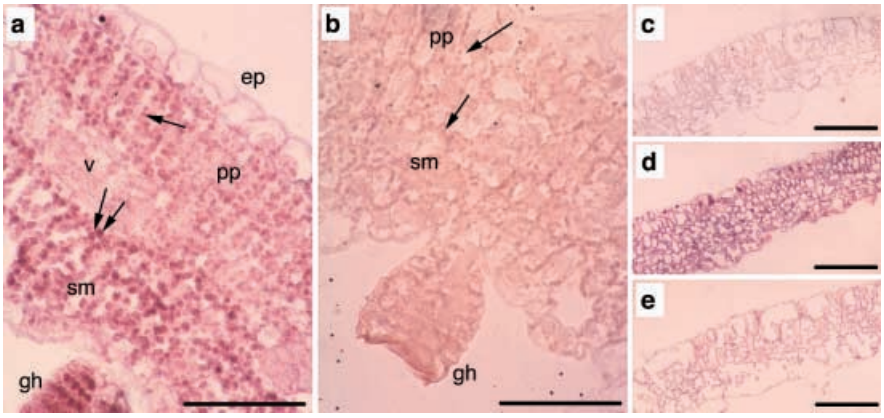


Fig. 3a–e. Validation of the IAA immunolocalization procedure. **a, b** Transverse sections of a young sunflower leaf with the complete procedure (**a**) or without primary antibody (**b**). *Arrows* indicate chloroplasts. Sections without secondary antibody incubation or without EDAC pre-fixation were similar to **b**. **c–e** IAA immunolocalization in longitudinal sections of strips of young leaves depleted of endogenous IAA (**c**) or loaded with 200 μM IAA (**d**) or 200 μM 2,4-D (**e**). *ep* Epidermis, *gh* glandular hair, *pp* palisade parenchyma, *sm* spongy mesophyll, *v* vascular tissue. Bars = 200 μm (**a, b**), 1.5 mm (**c–e**)

coloration of the sections occurred when the EDAC pre-fixation was omitted or when the complete fixation procedure was performed but without the primary or secondary antibody (Fig. 3b).

A second set of control experiments demonstrated the specificity of the immunological reaction for free IAA. Leaf sections were washed with a large excess of KPSC liquid medium for several hours in order to deplete them of endogenous IAA (McClure and Guilfoyle 1987). No specific coloration developed in such treated sections when submitted to the full immuno-cytochemical procedure (Fig. 3c). Sections that had been depleted of IAA but afterwards loaded with auxin by infiltration with a solution of IAA (200 μM) gave rise to intense coloration (Fig. 3d). In contrast, loading of the sections with the synthetic auxin, 2,4-D, resulted in a lack of coloration (Fig. 3e).

Spatial resolution

The staining reaction was sufficient to visualize the presence or absence of IAA in specific tissues. Coloration was absent in the epidermis and consistently weak in vascular tissue (Fig. 3a). In contrast, mesophyll cells always stained strongly for IAA. Moreover, the spatial resolution of the staining reaction allowed detection of IAA at the sub-cellular level. Indeed, within mesophyll cells, coloration was always very strong in chloroplasts (Fig. 3a). Ohmiya et al. (1990) previously reported localization of IAA in chloroplasts of leaf cells using immuno-chemical methods. These observations are also in agreement with results from wild-type or IAA-overproducing tobacco protoplasts (Sitbon et al. 1993) that demonstrated the existence of two separate sub-cellular IAA pools and suggested that free IAA is efficiently accumulated by chloroplasts.

The control experiments with sunflower leaf sections demonstrate that the employed monoclonal antibody is capable of specifically detecting IAA in sunflower tissues. The observed resolution allows drawing conclusions on the distribution of IAA between tissues, between different cell types, and even between organelles.

Distribution of IAA in in vitro-cultured embryos

Immature sunflower embryo explants (6 mm) were submitted to the immuno-cytochemical analysis immediately after excision (0 h), or after 14 and 24 h of culture on the embryogenic M12B medium as well as on the non-embryogenic M12 medium.

IAA distribution in freshly excised embryos

A weak signal was detected throughout the hypocotyl of the freshly excised embryos (Fig. 4a). The signal was slightly stronger in the root cap. The only strong signal detected was located in a narrowly delimited zone in the outermost cells of the root cap (Fig. 4b). The high local accumulation in a few cells may be responsible for the elevated average value measured in the mass-spectro-

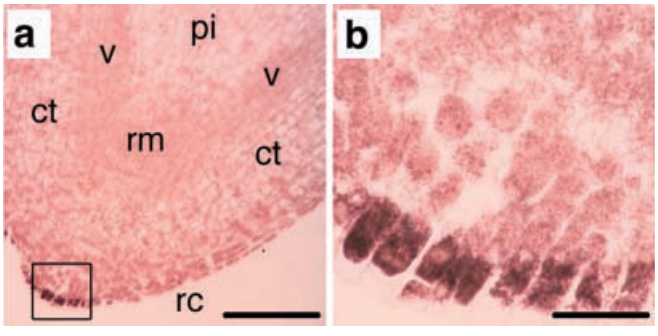


Fig. 4a, b. IAA distribution in a freshly excised sunflower IZE. **a** Signal detected within the hypocotyl. **b** Detail of the *boxed region* shown in **a** containing the strongly marked outermost cells of the root cap. *ct* Cortex, *pi* pith, *rc* root cap, *rm* root meristem, *v* vascular bundle. Bars = 500 μm (**a**), 25 μm (**b**)

metric analysis. This observation illustrates the complementarity of the two approaches. IAA accumulation in the root cap of immature sunflower embryos corroborates the results of Shi et al. (1993) that demonstrated the accumulation of IAA in root cap cells of *Z. mays*. More recently, Sabatini et al. (1999) showed indirectly, using the DR5::GUS auxin response reporter, that free IAA accumulates in the columella root cap cells of *Arabidopsis thaliana*.

IAA distribution after 14 h of culture

No signal for IAA was observed in explants that had been cultured for 14 h on the non-embryogenic M12 medium devoid of BAP (Fig. 5a). Even the characteristic accumulation of IAA in the outermost root cap cells of the freshly excised embryo had disappeared. No signal was detected in the morphogenic zone (Fig. 5b).

Explants cultured for 14 h on M12B medium showed a significant level of coloration throughout the embryo (Fig. 5c), including the morphogenic zone (Fig. 5d), indicative of the accumulation of IAA in response to the culture conditions. Two tissues accumulated IAA in a remarkable fashion above the generally increased level, i.e. the root cap and the root meristem (Fig. 5c, e). IAA also accumulated to a more than average level in the epidermal layer of the hypocotyl (Fig. 5c). In most of the marked cells, the signal was essentially limited to the cytoplasm (Fig. 5f). However, some isolated cells contained strongly marked nuclei (Fig. 5g). Such cells were preferentially found in the zone from which the future morphogenic reaction originates and in the wounded tissue resulting from the preparation of the explants for culture. This latter zone included the root meristem. In these zones, intense cell division activity would have occurred if explants had been cultured for a longer time. No marked nuclei were observed in tissues where any cell-division activity was soon to be expected. This phenomenon might be correlated with the re-activation of the cell cycle. Nuclear localization of IAA has already been described in meristematic cells in roots of peach seedlings (Ohmiya et al. 1990).

The present observation that IAA accumulated in characteristic areas under culture conditions favorable to the induction of cell divisions and subsequent somatic embryogenesis, but disappeared under conditions where no such induction occurred, suggests a correlation between the accumulation of IAA and either one of the mentioned subsequent events, i.e. re-entry into the cell cycle or induction of a novel morphogenic program.

IAA distribution after 24 h of culture

Explants cultivated for 24 h on M12 or M12B did not show any reaction indicative of the accumulation of IAA in any tissue or cell type on either medium (data not shown). The disappearance of the signal observed on

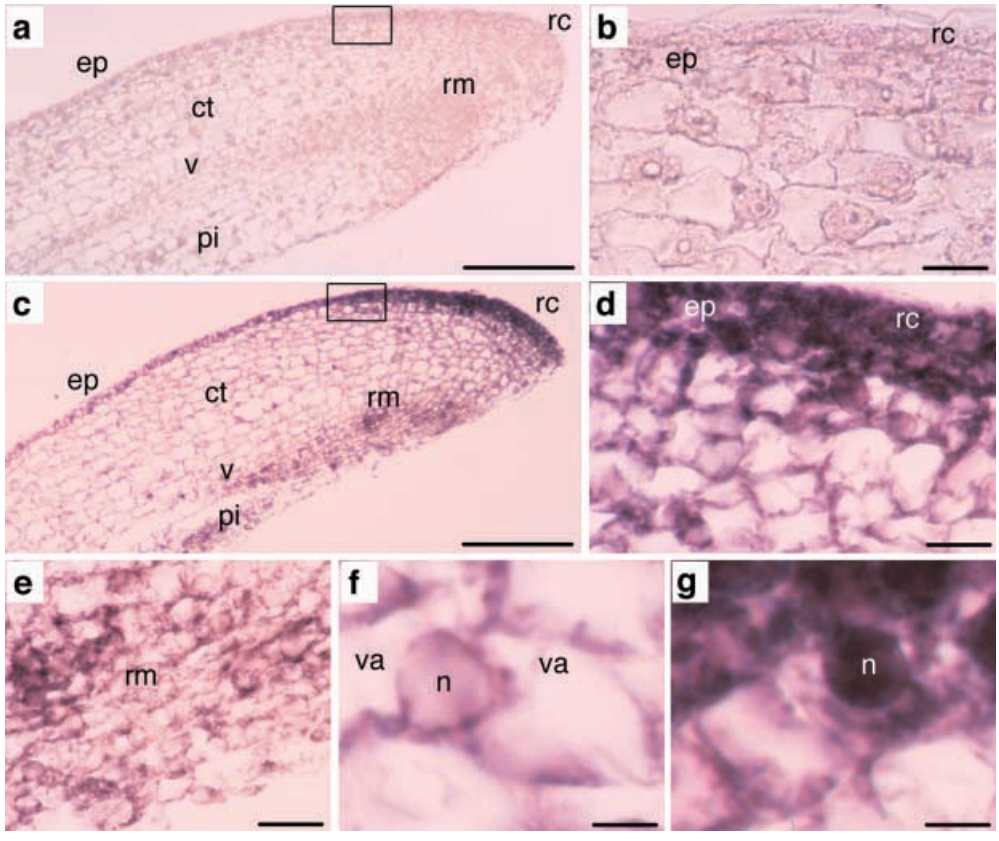


Fig. 5a–g. IAA distribution within sunflower IZE explants cultured for 14 h. **a, c** Explants cultured on the non-embryogenic medium M12 (**a**) or the embryogenic medium M12B (**c**). Morphogenic zones are indicated by a *rectangle*. **b, d** Details of the morphogenic zone of explants cultured on M12 (**b**) or M12B (**d**). **e–g** Details of **c**: root meristem (**e**); cytoplasmic signal observed in most cortical cells (**f**); strong nuclear signal observed in a cortical cell within the morphogenic zone (**g**). *ep* Epidermis, *ct* cortex, *n* nucleus, *pi* pith, *rc* root cap, *rm* root meristem, *v* vascular bundle, *va* vacuole. Bars = 1 mm (**a, c**), 25 μm (**b, d, e–g**)

embryogenic media is in agreement with the quantitative data (Fig. 2), which indicated a low overall content of IAA after 24 h of culture. The difference between the time points at 14 and 24 h is striking. Earlier studies have shown that explants cultured on M12B go very rapidly through a series of distinct initial steps, finally resulting in the development of somatic embryos (Bronner et al. 1994). Judging from morphological, histological and physiological data, the explants are clearly in distinct phases at 14 and 24 h after the onset of culture. First divisions specific to the embryogenic reaction become visible between 12 and 24 h of culture, and the clusters of dividing cells possess clear embryogenic characteristics at 48 h of culture (Bronner et al. 1994). If elevated levels of IAA were to explain the local induction of either cell proliferation or determination of the developmental fate (a specific event limited in space and duration), it would not be surprising that the accumulation of IAA at the future centers of cell division was transient. On a more global scale, induction of the embryogenic reaction in in vitro-cultured explants is often associated with a strong but transient external supply of auxin (Zimmerman 1993).

Conclusions

The induction period of somatic embryogenesis is clearly accompanied by a transient accumulation of IAA within the explant in response to the culture conditions. Pronounced differences in the IAA distribution pattern accompany the different phases of this reaction. It is clear that the observed variations in IAA concentration and distribution occurred as a reaction to the exposure to the cytokinin, i.e. BAP, present in the medium, as no IAA was detectable in the absence of BAP. It is as yet unclear which factors contribute to the differential accumulation in certain tissues at 14 h of culture and the dramatic decrease in IAA content after 24 h of culture, but these events are correlated in time with the morphogenic induction event. The observed changes strikingly resemble the experimental treatments (addition and removal of auxin) required for the induction of somatic embryogenesis in many classical somatic embryogenesis systems. However, a strong local IAA accumulation is clearly not sufficient for the induction of the expected morphogenic event, since IAA accumulation is not limited to the reactive cells. The whole root cap, the epidermis and the root meristem also showed a strong IAA accumulation although no morphogenic reaction occurs in these tissues. Accumulation of IAA to high levels may be a necessary condition for the sequence of events leading to the occurrence of the embryogenic reaction but (as yet unknown) signals of a different nature are required in addition to trigger the specific response in certain cells but not others. The accumulation of IAA in the nuclei of certain cells, but not others, also depended on the presence of cytokinin in the culture medium, and correlated well with locally occurring high concentrations of IAA and regions known to have increased mitotic activity. This correlation suggests that localized high (nuclear) concentrations of IAA, occurring under the influence of externally added cytokinin, might be involved in the induction of localized cell divisions, together with putative other specific factors.

To the best of our knowledge, no previous study has demonstrated the precise distribution of auxin in explants during the induction of somatic embryogenesis on the temporal and spatial levels, in relation to in vitro morphogenic events, although dynamic changes in the distribution of endogenous IAA have long been suspected to play a decisive role in the induction of such reactions (Mordhorst et al. 1997). The results presented here bring new and direct evidence for the widely accepted idea that an auxin pulse is among the first signals leading to the induction of somatic embryogenesis. This signal is likely to act in synergy with other signals, which still have to be identified.

Acknowledgements We thank Dr. J.L. Caruso (University of Cincinnati, Ohio, USA) for providing the IAA antibody and Dr. G. Jeannin (IBMP, Strasbourg, France) for many helpful suggestions and critical reading of the manuscript.

References

Bieleski WJ (1964) The problem of halting enzyme action when extracting plant tissues. Anal Biochem 9:431–442

Bronner R, Jeannin G, Hahne G (1994) Early cellular events during organogenesis and somatic embryogenesis induced on immature zygotic embryos of sunflower (*Helianthus annuus*). Can J Bot 72:239–248

Caruso JL, Pence VC, Leverone LA (1995) Immunoassay methods of plant hormone analysis. In: Davies PJ (ed) Plant hormones: physiology, biochemistry and molecular biology. Kluwer Academic Publishers, Dordrecht, pp 433–447

Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48:1493–1509

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Halperin W (1995) In vitro embryogenesis: some historical issues and unresolved problems. In: Davies PJ (ed) In vitro embryogenesis in plants. Kluwer Academic Publishers, Dordrecht, pp 1–16

Hutchinson MJ, Murch SJ, Saxena PK (1996) Morphoregulatory role of thidiazuron: evidence of the involvement of endogenous auxin in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium* × *horturum* Bailey). J Plant Physiol 149:573–579

Jeannin G, Charriere F, Bronner R, Hahne G (1998) Is predetermined cellular competence required for alternative embryo or shoot induction on sunflower zygotic embryos? Bot Acta 111:280–286

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol Biol 9:611–623

Moctezuma E (1999) Changes in auxin patterns in developing gynophores of the peanut plant (*Arachis hypogaea* L.). Ann Bot 83:235–242

Moctezuma E, Feldman L (1999) Auxin redistributes upwards in graviresponding gynophores of the peanut plant. Planta 209:180–186

Mordhorst AP, Toonen MAJ, de Vries SC (1997) Plant embryogenesis. Crit Rev Plant Sci 16:535–576

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–479

Ohmiya A, Hayashi T (1992) Immuno-gold localization of IAA in leaf cells of *Prunus persica* at different stages of development. Physiol Plant 85:439–445

Ohmiya A, Hayashi T, Kakiuchi N (1990) Immuno-gold localization of indole-3-acetic acid in peach seedlings. Plant Cell Physiol 31:711–715

Prinsen E, Redig P, Strnad M, Galis I, Van Dongen W, Van Onckelen H (1995) Quantifying phytohormones in transformed plants. Methods Mol Biol 44:245–262

Reinert J (1958) Morphogenese und ihre Kontrolle an Gewebekulturen aus Karotten. Naturwissenschaften 45:344–345

Sabatini S, Beis D, Wolkenfelt H, Murfett J, Guilfoyle T, Malamy J, Benfey P, Leyser O, Bechtold N, Weisbeek P, Sheres B (1999) An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell 99:463–472

Shi L, Miller I, Moore R (1993) Immunocytochemical localization of indole-3-acetic acid in primary roots of *Zea mays*. Plant Cell Environ 16:967–973

Sitbon F, Sundberg B, Olsson O, Sandberg G (1991) Free and conjugated indolacetic acid (IAA) contents in transgenic tobacco plants expressing the *iaaM* and *iaaH* IAA biosynthesis genes from *Agrobacterium tumefaciens*. Plant Physiol 95:480–485

Sitbon F, Edlund A, Gardeström P, Olsson O, Sandberg G (1993) Compartmentation of indole-3-acetic acid metabolism in protoplasts isolated from leaves of wild-type and IAA-overproducing transgenic tobacco plants. Planta 191:274–279

Steward F, Mapes M, Mears K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am J Bot 45:705–708

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5:1411–1423