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Auxin deprivation induces a developmental switch in maize somatic embryogenesis involving redistribution of microtubules and actin filaments from endoplasmic to cortical cytoskeletal arrays

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Abstract A developmental switch from non-polar pre-embryogenic units to polarized transition units in maize embryogenic callus is caused by auxin deprivation from the culture medium. This switch is accompanied by cytoskeletal rearrangements in embryogenic cells. An immunofluorescence study revealed prominent endoplasmic microtubules and actin filament meshworks radiating from the nuclear surfaces in pre-embryogenic cells growing on medium supplemented with auxin. On the other hand, parallel-organized cortical microtubules and cortical actin filament networks are inherently associated with polarized embryogenic cells of transition units growing on medium without auxin. These results indicate that fine-tuning of the dynamic equilibrium between endoplasmic and cortical cytoskeletal arrays is important for progress in somatic embryogenesis.

Keywords Actin filaments · Embryogenic callus · Microtubules

Abbreviations *AGP:* Arabinogalactan protein · *BSA:* Bovine serum albumin · *2,4-D:* 2, 4-Dichlorophenoxyacetic acid · *FITC:* Fluorescein isothiocyanate · *MS:* Murashige and Skoog medium · *PBS:* Phosphate-buffered saline · *SB:* Stabilizing buffer

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Introduction

Somatic embryogenesis in maize proceeds through well-defined cellular structures and growth patterns. In early embryogenic stages, both induction of cell division and suppression of cell growth are necessary. On the other hand, polarized cell growth is required for advanced embryogenic stages (Šamaj et al. 1995, 1997).

Early embryogenic stages (so-called pre-embryogenic units) are dependent on exogenous auxin, while development of subsequent stages (transition units and somatic embryos) requires auxin deprivation from the culture medium. Exogenous auxin is important for callus cells in order to confer embryogenic potential and to induce mitotic divisions, but auxin deprivation is necessary for further progress of embryogenic morphogenesis. We assume that the cytoskeleton plays a crucial role in these developmental switches driving somatic embryogenesis.

Most differentiated plant cells have their microtubules at cell peripheries organized as individual or bundled cortical microtubules. These microtubules determine the orientation of cellulose microfibrils, both polymers providing structural support for polarized cell growth (for review see Barlow and Baluška 2000). Besides cortical microtubules, plant cells also assemble endoplasmic microtubules, which radiate from nuclear surfaces towards peripheries of plant cells (for maize root cells see Baluška et al. 1992, 1996a). Unlike cortical microtubules, endoplasmic microtubules do not form bundles. The minus ends of these microtubules are attached to the microtubule-organizing nuclear surface (Lambert 1995; Baluška et al. 1997), whereas the plus ends dynamically explore the cytoplasmic space (Holy and Leibler 1994). This spatial microtubule arrangement suggests that endoplasmic microtubules might be involved in some sort of signaling between the nucleus and cell periphery (Barlow and Parker 1996; Baluška et al. 1997, 2000). In tip-growing higher plant cells, such as pollen tubes and root hairs, fine networks of microtubules interconnect subapical nuclei with the apical tip zone, which is enriched with F-actin (Lloyd et al. 1987; Baluška et al. 2000). This

feature seems to be essential for the sustained signal-mediated tip growth of these cells (Li et al. 1997; Staiger 2000; Hepler et al. 2001; Šamaj et al. 2002).

Radiating endoplasmic microtubules are prominent in plant cells treated with taxol (Baluška et al. 1997) and inhibitors of both protein synthesis (Mineyuki et al. 1994; Baluška et al. 1995) and protein kinases/phosphatases (Baskin and Wilson 1997). They are also abundant in *Arabidopsis* mutants with affected cell growth polarity and morphogenesis (Hauser et al. 1995; Traas et al. 1995). Numerous endoplasmic microtubules are present in cells treated with auxin (for maize roots see Baluška et al. 1996b) or subjected to diverse stresses including cold (Baluška et al. 1993), heat (Hause et al. 1993; Gervais et al. 2000), mechanical stress (Caumont et al. 1997), and aluminium (Sivaguru et al. 1999). Similarly, during sporogenesis (Dickinson and Sheldon 1984), endosperm development (Brown and Lemmon 1988; Brown et al. 1994), and *Rhizobium* infection (Timmers et al. 1999), centered nuclei organize almost all microtubules in the form of radial arrays. In all these situations, cortical microtubules are usually depleted and/or disassembled. However, little is known about cytoskeletal organization of embryogenically competent cells during embryogenic induction and progress (Caumont et al. 1997).

Previously we have studied cell wall arabinogalactan proteins (AGPs) in maize embryogenic callus and discussed their function in cell adhesion and patterning, as well as in signaling cell position and fate within embryogenic callus (Šamaj et al. 1999a, b). It was suggested that the cell wall – plasma membrane – cytoskeleton continuum plays a crucial role in plant morphogenesis. In this work we focused on cytoskeletal arrangements in embryogenic cells of pre-embryogenic and transition units representing two critical stages in which the growth pattern is switched from non-polarized to polarized mode. We report that perinuclear microtubules and actin microfilaments are important for induction of the embryogenic pathway in pre-embryogenic single cells and units, while cortical microtubules and actin filaments are necessary for polarized cell growth in transition units.

Materials and methods

Plant material and tissue culture

Maize embryogenic callus culture was induced from immature embryos of maize hybrid, genotype 04Z035070×Microsperma, having high regeneration potential. The callus lines were maintained on agar-solidified (0.7%) MS medium supplemented with 12.7 m*M* 2,4-dichloro-phenoxyacetic acid and 88 m*M* sucrose in darkness at 24°C, and subcultured every 3 weeks (for details see also Šamaj et al. 1995, 1999a).

Histology

Callus was fixed, embedded and sectioned exactly as described previously (Šamaj et al. 1999a). In summary, callus clumps were fixed in 100 m*M* phosphate-buffered saline (PBS, pH 7.2) containing 5% glutaraldehyde for 5 h and postfixed with 1% OsO_4 for 1 h. Samples were washed with PBS, dehydrated in a graded acetone series and embedded in Durcupan ACM resin (Fluka, Buchs, Switzerland). Semithin sections were prepared using a Reichert OM U3 ultramicrotome (Reichert, Vienna, Austria) and double-stained with toluidine blue and basic fuchsin in order to visualize intracellular structures and cell walls respectively.

Immunofluorescence and microscopy

Samples for cytoskeleton immunolabelling were prepared from callus cultured for 2–10 days on medium containing exogenous auxin or medium deprived of exogenous auxin. Callus clumps were directly fixed on plates with 3.7% formaldehyde in stabilizing buffer (SB, 50 m*M* piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) buffer, 5 m*M* $MgSO_4$, 5 m*M* EGTA, pH 7.0) for 2 h at room temperature (first 20 min with vacuum infiltration). After washing in SB and PBS(100 m*M*, pH 7.2) samples were dehydrated in a graded ethanol series diluted with PBS. Tissue was embedded in Steedman's wax prepared from polyethylene glycol 400 distearate and 1-hexadecanol (9:1) mixed at 37°C and left to solidify at room temperature. Semithin sections (7–15 μm thick) were mounted on slides coated with glycerol albumin (Serva, Heidelberg, Germany) or Biobond adhesive (BioCell, Cardiff, UK). Sections were then dewaxed in absolute ethanol, rehydrated in a graded ethanol series and rinsed in SB and PBS containing 5% bovine serum albumin (BSA). Subsequently, sections were incubated with monoclonal anti-α tubulin (Amersham, Buckinghamshire, UK) or anti-actin (ICN Biomedicals, Costa Mesa, Calif., USA, clone C4) antibodies, both diluted 1:200 in PBS containing 1% BSA, for 1 h at room temperature. After washing in PBS containing 1% BSA, they were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgGs diluted 1:100 in the same buffer. After rinsing in PBS, the sections were treated with 0.01% toluidine blue (diluted in PBS), which diminishes autofluorescence of tissues. Sections were finally mounted in antifade mountant containing glycerol and *p*-phenylenediamine. As negative control, the primary antibody was omitted. Epifluorescence was examined with Axiovert 405 M or Axioplan 2 microscopes (Zeiss, Oberkochen, Germany) equipped with epifluorescence, standard FITC exciter and barrier filters (BP 450–490, LP 520). Photographs were taken on Kodak T-Max films rated at 400 ASA. Confocal images of immunofluorescently labelled samples were collected from 15 μm sections using Leica confocal microscope TCS4D (Leica, Heidelberg, Germany). Some digital images were captured as red-green anaglyphs in order to ensure better spatial resolution of the cytoskeleton, processed with Photoshop (Adobe) and Corel Draw (Corel) and printed on an Epson Stylus Color 740 printer.

Results

Individual pre-embryogenic cells and units are formed at the peripheries of maize embryogenic callus cultures when these are cultured on MS medium supplemented with 2,4-D. These pre-embryogenic units are mostly non-polar and globular in form; they are formed from meristematic cells surrounded by callus tissue (Fig. 1A). Outer cells of these embryogenic clumps are loosely attached to each other, and their cell walls possess AGPs (Šamaj et al. 1999a). Embryogenesis proceeds further only after auxin deprivation from the culture medium. Without supplied auxin, most pre-embryogenic units convert into transition units within 4–6 days. These are polarized structures composed of tightly arranged meristematic cells on the apical pole and suspensor-like elongated cells on the basal pole (Fig. 1B).

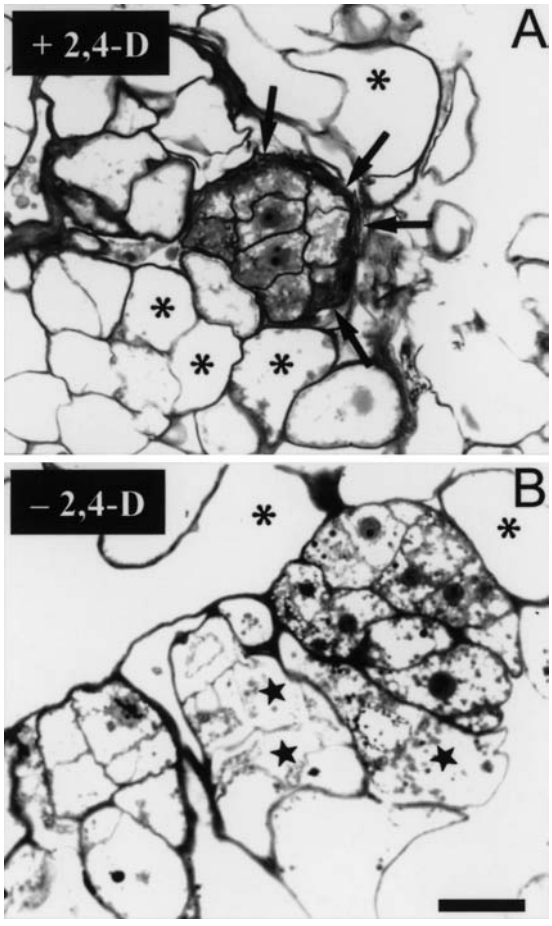

Fig. 1 **A** Histology of pre-embryogenic units developed in maize callus cultured on media supplemented with auxin. **B** Histology of transition units cultured for 5 days on media without auxin. *Arrows* in **A** point to thick outer cell walls, which surround, and make loose contact with, outer pre-embryogenic cells. *Asterisks* mark non-morphogenic callus cells. *Stars* in **B** indicate suspensor-like parenchyma cells. *Bar* in **B** is valid for both images **A** and **B** and represents 40 μm

The cytoarchitecture of pre-embryogenic units indicates that their cells are at an activated stage induced by the presence of auxin. The microtubular cytoskeleton of these embryogenically competent cells is dominated by numerous endoplasmic microtubules, which radiate from nuclear surfaces towards cell peripheries where they are connected with networks of cortical microtubules (Fig. 2A–D). In contrast to the prominent endoplasmic microtubules, cortical microtubules are clearly less abundant in embryogenic cells at this stage and they are organized into fine networks (Fig. 2C, D). On the other hand, adjacent non-embryogenic callus cells, which could serve as good control, display well-developed cortical arrays of microtubules but no detectable endoplasmic microtubules (Fig. 2D).

After auxin deprivation, the most prominent cytoskeletal change in the embryogenic cells of the transition units is assembly of well organized and parallel-oriented cortical microtubules, the feature most pronounced in meristematic cells of the apical pole (Fig. 2E, F) cultured on medium without exogenous auxin for 5 days. Concomitantly, endoplasmic microtubules surrounding nuclei become randomized and depleted in these cells when compared with pre-embryogenic stages (Fig. 2F). Cortical but not endoplasmic microtubules could be also visualized in suspensor-like cells at the basal pole of transition units (Fig. 2E) and in some callus cells (data not shown).

In auxin-activated pre-embryogenic cells, actin is organized in the form of a meshwork which is enriched around nuclear envelopes and protein bodies distributed within the cytoplasm and protrudes towards cell peripheries (Fig. 3A, B). Auxin deprivation for 5 days also caused re-arrangements in the actin cytoskeleton. In embryogenic cells of transition units, actin filaments formed perinuclear baskets (Fig. 3C, D). With this exception, cortical actin filaments are associated with cell peripheries and plasma membranes of auxin-depleted cells (Fig. 3C, D).

Discussion

In maize callus, addition of 2,4-D to the culture medium leads to embryogenic induction of callus cells (Šamaj et al. 1995) accompanied by secretion of a distinct subset of AGPs into the outer cell walls of embryogenic cells (Šamaj et al. 1999a, b). During this process, nuclei are actively positioned into the centers of embryogenically competent cells. Similarly, the nuclei of embryogenic microspores assume a central position during acquisition of embryogenic competence in these cells (Gervais et al. 2000). Both microtubules and actin filaments radiating from nuclear surfaces are involved in the centering of the nuclei and also in maintaining and sensing this central nuclear position via interactions with the cortical cytoskeleton (Grolig 1998; Volkmann and Baluška 1999; Baluška et al. 2000; Collings and Allen 2000). Immunofluorescence data presented in this study show that prominent radiating perinuclear microtubules and actin filaments are characteristic of individual pre-embryogenic cells and units of maize callus. This cytoskeletal arrangement in auxin-induced embryogenic cells seems to be related to their embryogenic competence because no obvious endoplasmic cytoskeleton was detected in surrounding non-embryogenic callus or suspensor-like cells in later embryogenic stages. Similar organization of microtubules and actin filaments is characteristic also for microspores induced into embryogenesis (Hause et al. 1993; Gervais et al. 2000).

If we consider the structural continuity within the cell wall – plasma membrane – cytoskeleton complex, then the above cytoskeletal arrangements of pre-embryogenic cells could be related to their unique cell-to-cell contacts and the composition of cell walls. Embryogenic cells of pre-embryogenic units have loose contacts (Šamaj et al. 1995) and contain AGPs which are believed to have signalling properties (Šamaj et al. 1999a, b). Here we show that the same cells assemble abundant endoplasmic

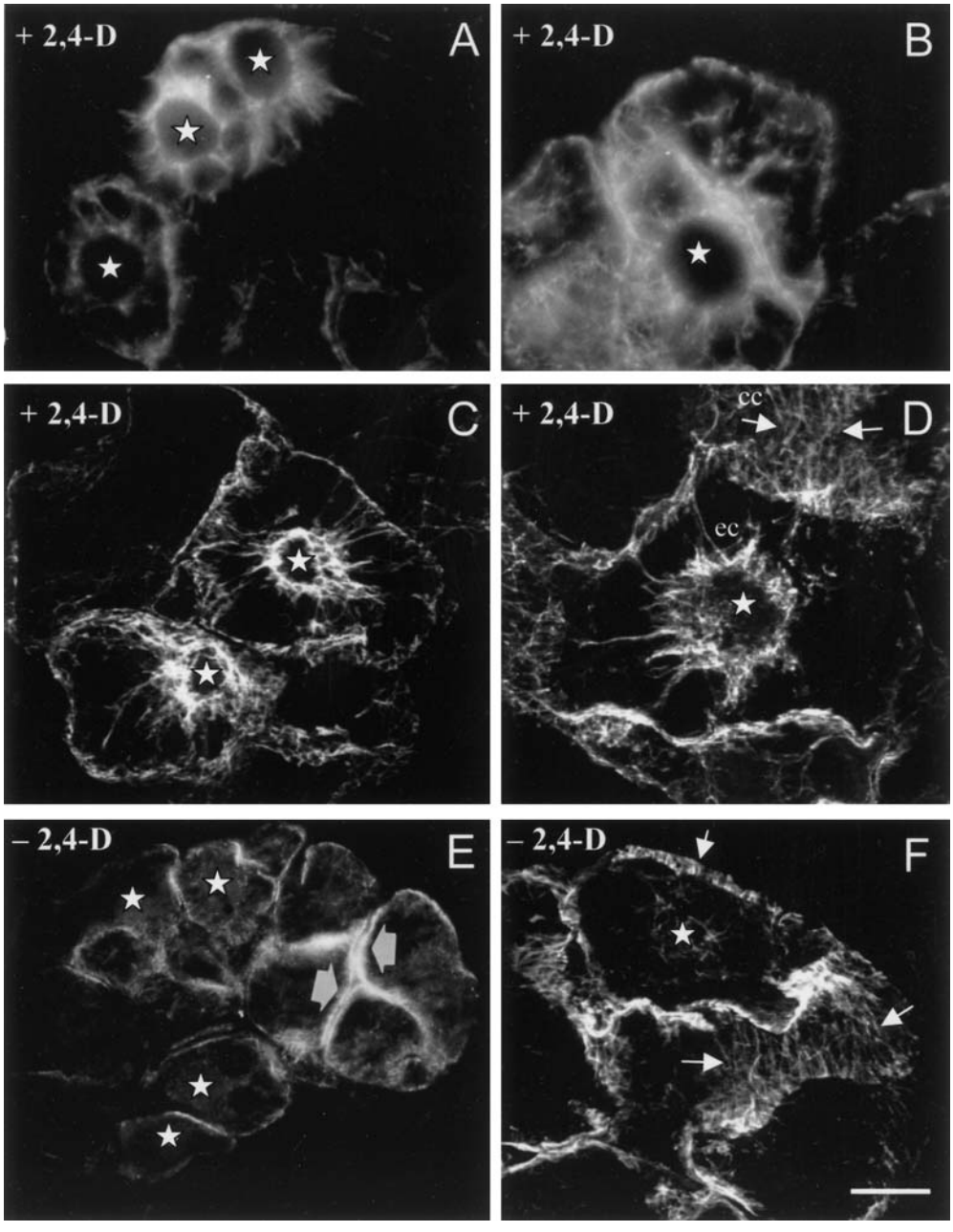

Fig. 2 Immunofluorescence detection of microtubules in cells of pre-embryogenic (**A**–**D**) and transition (**E**, **F**) units of maize callus cultured on media supplemented with auxin (**A**–**D**) or without auxin for 5 days (**E**, **F**). Positions of nuclei are indicated with *asterisks*. Note that conspicuous endoplasmic microtubules that radiate from nuclei are characteristic of pre-embryogenic cells (**A**–**D**), while these microtubular arrays are depleted and randomized in cells of transition units, appearing as shorter microtubules surrounding the nucleus (**F**). Note that cortical microtubules are arranged in the form of fine networks within pre-embryogenic cells (*ec*) in **D**, in contrast to dense arrays of cortical microtubules in non-embryogenic callus cells (*cc* and *arrows*) in **D**. After auxin deprivation for 5 days, embryogenic cells of transition units assemble dense cortical microtubules (*arrows* in **E** and **F**), while endoplasmic perinuclear microtubules are depleted or randomized both in meristematic and suspensor-like cells (*asterisks* in **E** and **F**). *Bar* in **F** is valid for all images and represents 12 μm for **B** and **D**; 15 μm for **A**, **C** and **F**; and 25 μm for **E**

cytoskeleton but that their cortical cytoskeleton is rather depleted. We propose that both radiating perinuclear microtubules and actin filaments allow rapid signalling to the nucleus, which enables these cells to respond to changing extracellular stimuli including different levels or absence of exogenous auxin. This suggestion is supported by data from other embryogenic and non-embryogenic systems. For instance, sunflower protoplasts cultured in liquid medium form loosely associated cells and do not embark on the embryogenic pathway. However, when the same cells are embedded in agarose, they start to deposit callose at their reforming cell walls, assemble numerous perinuclear microtubules, and enter the embryogenic pathway (Caumont et al. 1997). It would be interesting to know whether these callosic cell walls also accumulate some AGPs. Another example is that rhizobia can trigger arrangement of endoplasmic microtubules in non-dividing outer cortical cells of legume roots (Timmers et al. 1999).

Further morphogenesis in maize embryogenic callus is induced after auxin deprivation; the preferred response to this is the assembly of cortical microtubules and, to some degree, actin microfilaments at the peripheries of embryogenic apical cells. Previously we reported that embryogenic cells of transition units are tightly arranged (Šamaj et al. 1995). Abundant and parallel-oriented cortical microtubules could therefore be important for their tight cell–cell contacts and coordinated cell expansion, which also allows progressive polarization of transition units. In agreement with our data, abundant assembly of cortical microtubules was shown to be characteristic for cells in

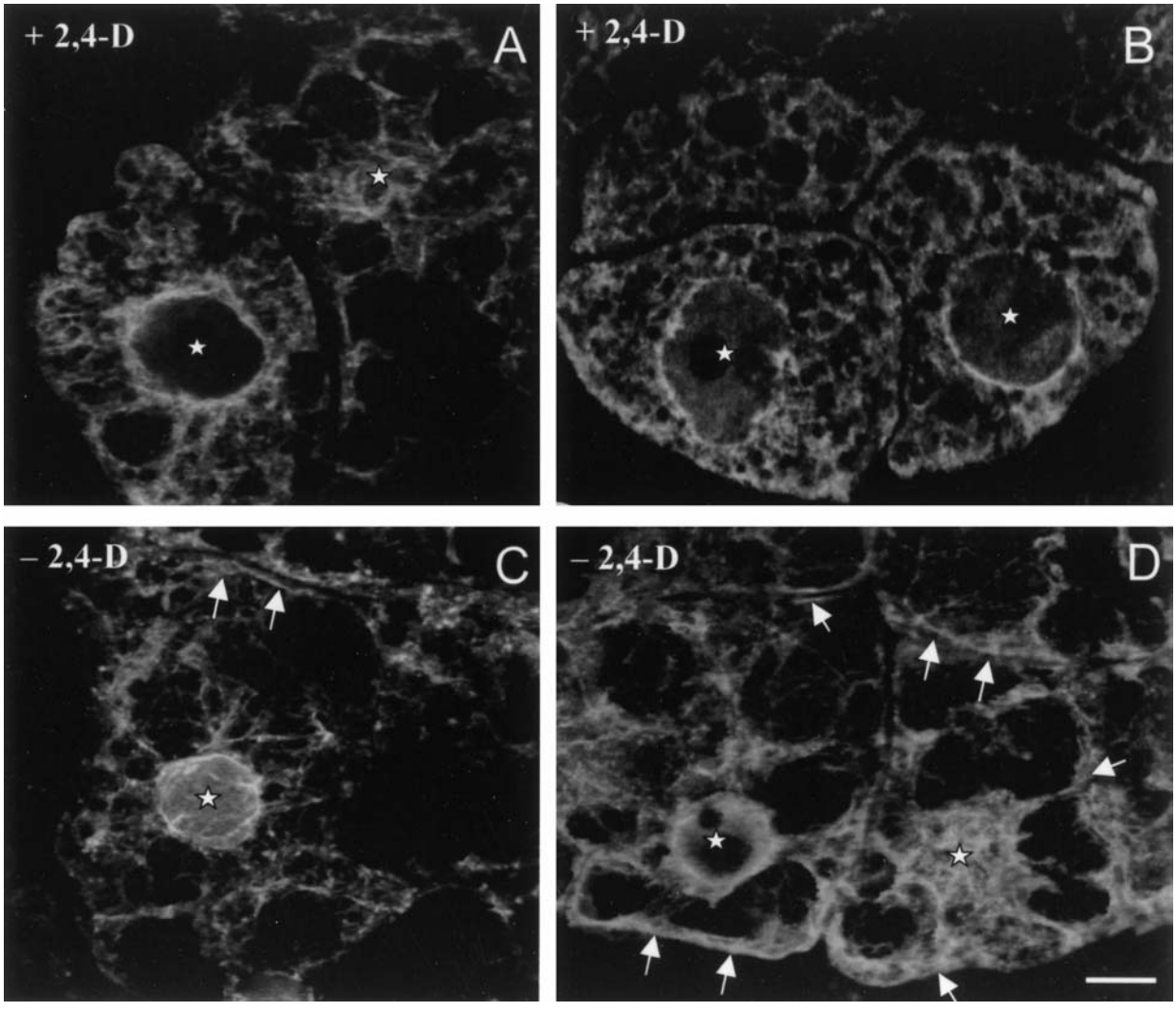

Fig. 3 Immunofluorescence localization of actin cytoskeleton in cells of pre-embryogenic (**A**, **B**) and transition (**C**, **D**) units of maize callus cultured on media supplemented with auxin (**A**, **B**) or without auxin for 5 days (**C**, **D**). Nuclei are indicated by *asterisks*. Actin filaments of pre-embryogenic cells are organized in form of meshworks of shorter filaments, enriched around nuclei and protein bodies and pervading the whole cytoplasm up to the cell peripheries (**A**, **B**). Actin filaments in meristematic cells of transition units form perinuclear baskets and cortical actin networks associated with plasma membrane (*arrows* in **C**, **D**). *Bar* in **D** is valid for all images and reperesents 10 μm

embryogenic cultures of black spruce (Tautorus et al. 1992). Cortical microtubules were also observed in regions adjacent to the new cross-walls in cellular clumps of alfalfa (Meijer and Simmonds 1988).

Taken together, these data indicate that perinuclear microtubules and actin filaments connected to fine cortical microtubule and actin networks are prominent in early embryogenic cells in which a polar axis has not yet been established. We suggest that this unique cytoskeletal arrangement is relevant to the embryogenic competence of these cells. On the other hand, densely organized cortical microtubules and cortical actin filaments are important for morphogenetic development, tight cell-to-cell contacts and, especially, for the polarized growth of transition units. We conclude that the most critical switch in the embryogenic development from non-polar to polar structures is accompanied by re-arrangements of cytoskeletal arrays, which might reflect concomitant changes in cell wall organization (Šamaj et al. 1999a, b). These changes are specific for embryogenic cells since they do not occur in non-embryogenic callus cells. Dynamic equilibrium between endoplasmic and cortical microtubules and actin filaments within embryogenic cells appears to be especially important for progression in somatic embryogenesis.

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