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IMMUNO-GOLD LABELLING OF TUBULIN IN ULTRATHIN CRYOSECTIONS OF CULTURED CARROT CELLS

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SUMMARY

Ultrathin cryosections of somatic embryos from carrot suspension cultures were labelled for tubulin with colloidal gold and post-embedded in London Resin White. Good contrast, ultrastructure and antigenicity were preserved. The distribution of immuno-gold labelled tubulin was studied in individual cell organelles.

The highest densities of gold particles were found in the cytoplasm, especially the cortical cytoplasm. Nuclei, and notably the nucleoli showed a less intense but positive labelling. The labelling of plastids and mitochondria might be aspecific but it cannot be excluded that some tubulin is present here. Vacuoles and cell walls were negative. Control experiments, in which at least the first antibody was omitted, were negative too.

Although the results have to be interpreted carefully, they indicate that differences exist in the distribution of tubulin amongst and within individual cell organelles. The method, therefore, might be useful to study developmental changes in the microtubular cytoskeleton at a high resolution level.

1. INTRODUCTION

The visualization of single microtubules has been realized with the electron microscope since 1963 (LEDBETTER & PORTER 1963) but recently immunocytochemical techniques greatly stimulated their detection with both light and electron microscopy (for a survey, see e.g. DUSTIN 1984). In plants, microtubules are known to manifestate in various cytoskeletal arrangements throughout the cell cycle and during the subsequent stages of cell differentiation. Additionally, tubulin subunits might occur in various concentrations during the cell cycle. Cortical, cytoplasmic and spindle microtubules were detected and their functions were discussed (DE MEY et al. 1982; HAHNE & HOFFMANN 1985; LLOYD 1982; SCHROEDER et al. 1985; WICK et al. 1985). In these studies, however, little attention was paid to determine fluctuations in the total quantity of tubulin.

Recently, VAN LAMMEREN et al. (1985) described a cytofluorometric method to visualize and quantify fluorochrome-labelled microtubules and tubulin subunits in embedment-free semi-thin sections of plant cells. The resolution of these measurements is, however, limited because the quantification is based upon the intensity of the total light emission of an area of about 100 μm^2 of the fluorochrome-labelled section. The fluorescence from individual organelles therefore

could not be determined. Colloidal gold labelling of tubulin on ultrathin sections and observation by electron microscopy might overcome this problem and allow a high-resolution study of the tubulin distribution inside the cellular organelles.

In this paper the distribution of labelled tubulin is shown, using electron micrographs of ultrathin frozen sections obtained from embryogenic cell aggregates from *Daucus carota* L. suspension cultures. These cultures are very suited to study developmental changes in the cytoskeleton of somatic embryos because of the high frequency of regeneration and the possibility to synchronize the embryogenic stages (AMMIRATO 1983).

2. MATERIALS AND METHODS

2.1. Cell cultures

Suspension cultures of a *Daucus carota* L. embryogenic cell line were used. Culture conditions and induction of embryogenesis were essentially as described earlier (GIULIANO et al. 1983). Small aggregates of cells were harvested at 5 days after the induction of embryogenesis (d.a.i.).

2.2. Reagents

The IgG fraction of the monoclonal anti-β-tubulin (type MON SK 4002, Sanbio, Uden, Holland) was used. This antibody was raised in mouse cells against rat brain tubulin. Both the unconjugated and the conjugated antiglobulin, raised in swine against mouse (SwaM resp. SwaM/FITC) were obtained from Nordic, Breda, Holland. Protein A-gold (pAG, 7 nm) was supplied by Janssen Pharmaceutica, Beerse, Belgium.

2.3. Immunofluorescence

Fixation, dehydration and embedment of the cell clumps was done essentially as described by VAN LAMMEREN et al. (1985). Semithin (3 μm) sections were labelled with the anti-β-tubulin (dilution 1:100, 45 min, 37 °C), and with SwaM/FITC (1:25, 45 min, 37 °C). Further procedures were as described by VAN LAMMEREN et al. 1985.

2.4. Ultracryomicrotomy

Cell aggregates were fixed in 3% paraformaldehyde + 0.25% glutaraldehyde in microtubule-stabilizing-buffer (MSB) according to HAWES (1985). After fixation they were washed twice in MSB, 30 min, 20 °C. The cells were then immersed in 0.1 M sucrose in phosphate-buffered saline (PBS) for 2 × 1 h at 20 °C, then in 1.0 M sucrose in PBS for 2 × 1 h at 20 °C, and finally in 2.3 M sucrose in PBS, overnight at 4 °C. They were mounted on copper stubs with some 2.3 M sucrose, quickly frozen in a liquid nitrogen slush, and ultrathin sectioned at −80 °C using an LKB Nova ultracryomicrotome. The frozen sections were picked up on a drop of 2.3 M sucrose, thawed, and transferred onto ionized Formvar and carbon-coated 200 mesh grids. The grids were floated, sectionside down, on a 2% gelatin solution in PBS before immuno-gold labelling.

2.5. Immuno-gold labelling

All steps were carried out at room temperature. The mounted sections were first washed on drops of 0.02 M glycine in PBS. Quenching of aspecific binding was performed by floating the sections successively on drops of 0.1 M NH_4Cl for 5 min, on freshly prepared $NaBH_4$, 0.5 mg/ml for 5 min, and on 0.5% BSA, 3 times 5 min, all diluted in PBS. All reagents, further described, were prepared in PBS, supplemented with 0.5% BSA, and centrifuged at 10,000 g for 5 min directly before use.

Sections were incubated with 10 µl anti-tubulin (1:5 dilution, 45 min). After three rinses in buffer, 5 min each, they were incubated with 10 µl SwaM-antiglobulin (1:400, 45 min). After that, the sections were rinsed three times in buffer, supplemented with 0.2% gelatin, and incubated with 10 µl pAG (1:250, diluted in the same buffer, 45 min). The pAG-solution was not centrifuged. After three washes in PBS and distilled water, the grids were postfixed in 2% OsO_4 in aquadest, washed and post-stained in neutral and aqueous uranylacetate according to TOKUYASU (1978). The sections were dehydrated and post-embedded in London Resin White (Agar Aids, medium grade), as described before (KELLER et al. 1984). The grids were examined in a Philips EM 301 at 60 kV.

3. RESULTS

Microtubular cytoskeletons, present in somatic embryos which were induced during culture, are shown in the *figs. 1a* and *1b*. By means of immunofluorescence labelling many microtubules are visible in the globular stage of development (*fig. 1a*). Some of them run throughout the central cytoplasm but mostly they are concentrated near the cell membranes (*fig. 1a*, thin arrows). Criss-cross patterns are frequently observed in the isodiametric cells of the suspension culture itself (*fig. 1a*, thick arrows). In the torpedo-shaped embryos, present at about 12 d.a.i., most cells of the hypocotyl have elongated. In these cells the cortical microtubules are often found in parallel arrangements transverse to the direction of cell elongation (*fig. 1b*, thick arrows). When they are cut perpendicularly only fluorescent spots accentuate their presence (*fig. 1b*, thin arrows). Because of the high density of microtubules they cannot be distinguished individually as can be done with ultrathin Epon sectioning (*fig. 1c*, arrows).

First attempts, using ultrathin frozen sections, initially were unsuccessful. Poor contrast and a bad preservation of ultrastructure were obtained. When these cryosections, however, were post-embedded in London Resin White, as was pioneered by KELLER et al. (1984) and described also for plant cells by GREENWOOD et al. (1984) and GREENWOOD & CHRISPEELS (1985), much progress was made. *Fig. 1d* shows that, with this procedure, good contrast is achieved. Many organelles are well-preserved and easy to recognize; for example, the nuclear envelope might be indicative for this.

The labelling of ultrathin cryosections of embryonic cells with various antibodies against tubulin is shown in *fig. 2*. Best labelling was observed after a three-step procedure (*fig. 2a*). When the antiglobulin was omitted during labelling

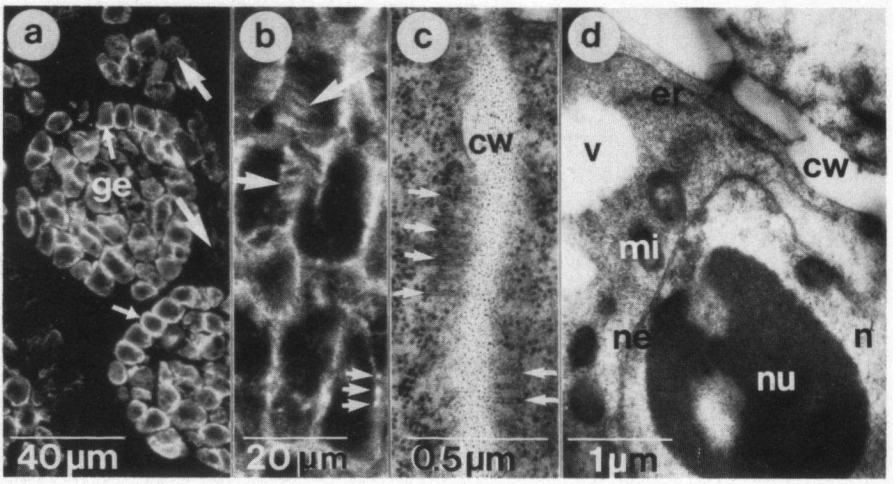

Fig. 1. a. Semi-thin (3 µm) sections of cells from a carrot culture at 5 days after the induction of somatic embryogenesis. Embedment in polyethylene glycol and FITC-labelling of embedment-free sections. Some globular embryos are visible (ge). Note the concentration of FITC-labelled microtubules near the cell membranes in these embryos (thin arrows). The other cell aggregates show microtubules arranged in criss-cross patterns (thick arrows); b. Detail of a torpedo-shaped somatic embryo 12 days after induction of embryogenesis. Same preparation method as described under a. Parallel running microtubules are visible as either fluorescent lines (thick arrows) or spots (small arrows); c. Ultrathin Epon section of a cell aggregate at 7 days after embryo induction. Cortical microtubules can be distinguished individually (arrows). Glutaraldehyde-osmiumtetroxide fixation; d. Ultrathin cryosection of a cell from a globular embryo at 5 days after embryo induction. Post-embedment in London Resin White. There is a good preservation of the general ultrastructure; many organelles, e.g. nucleus (n), nucleolus (nu), vacuoles (v) and mitochondria (mi) can be recognized. Also the cell wall is visible (cw). Note the distinct nuclear envelope (ne).

(*fig. 2b*), the intensity of the labelling was reduced about twice which indicates that the use of the SwaM-antiglobulin improves the binding of pAG particles (Paul van Bergen en Henegouwen, pers. comm.). The control experiments, using frozen sections from the same specimen, were negative, indicating that both binding of the SwaM intermediate (*fig. 2c*) and of the pAG complex (*fig. 2d*) to cellular structures was neglectable. The subcellular distribution of gold particles is summarized in *table 1*.

4. DISCUSSION

To quantify the microtubular distribution at the organelle level, the labelling of the tubulin protein itself is the method of choice. This is caused by the resolution limits of the immunofluorescence method. Ultrathin serial sectioning, although theoretically possible, would be a tremendous task. Besides that, microtubules might partially dissociate during the dehydration and embedding procedures. Ultracryomicrotomy can overcome these problems and, moreover, improve the accessibility of the tubulin and the preservation of antigenicity (see,

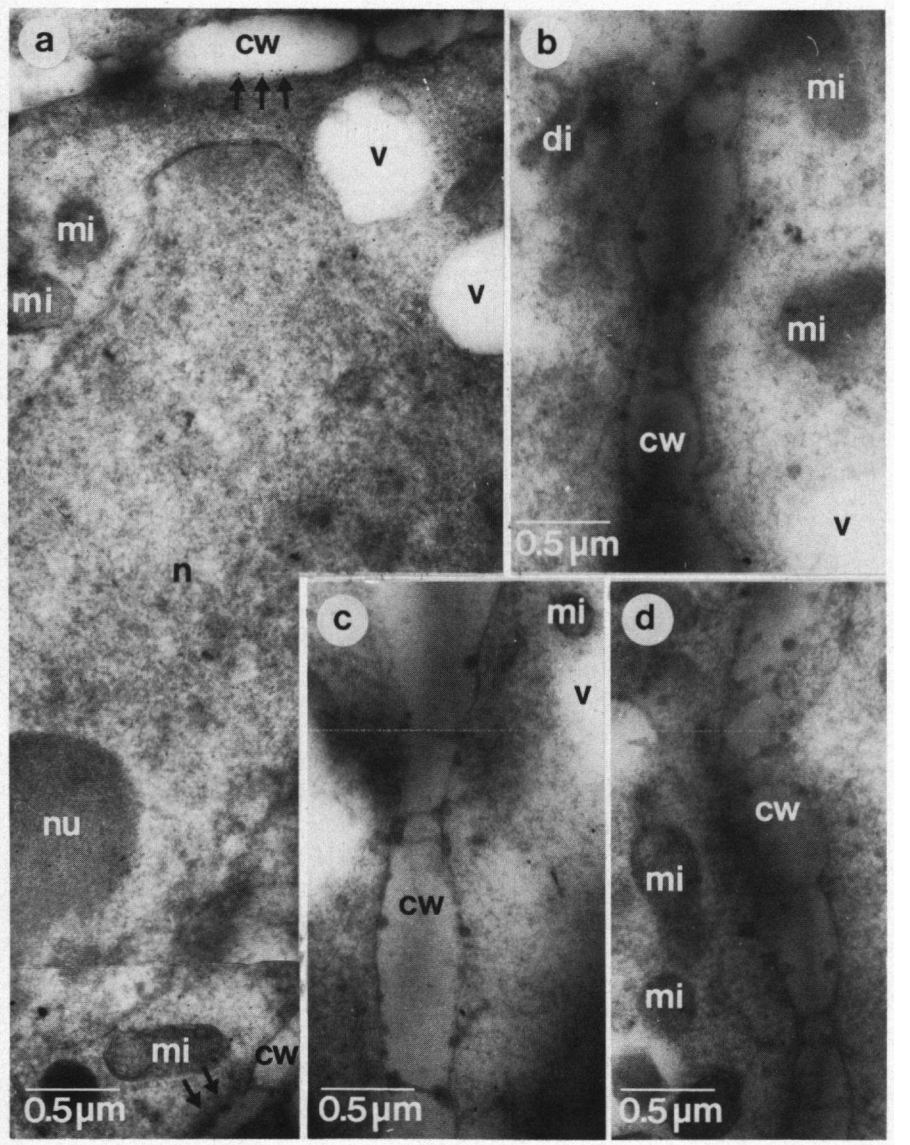

Fig. 2. Ultrathin cryosections of somatic carrot embryos at the globular stage of development (5 days after embryo induction) labelled for tubulin with various antibodies and post-embedded in London Resin White. a. Tubulin was labelled by the indirect method using a three-step procedure: antibody-antiglobulin-pAG. The dense labelling in the cortical regions of the cell is evident (arrows). b. Tubulin was labelled by a two-step procedure omitting the antiglobulin. Note the differences in the intensity of the labelling in the various cell areas; di, dictyosome. c. Control section for the three-step labelling procedure; the monoclonal antibody was omitted. d. Control section for the two-step procedure; labelling without antibody and antiglobulin.

Table 1. A representative distribution of gold-labelled tubulin in an ultrathin cryosection of a somatic embryo cell of carrot. For each organelle the number of gold particles is expressed per square micrometer. In the control the tubulin antibody was omitted.

Organelle	Total number of gold particles/organelle		Organelle area (μm^2)		Number of gold particles/μm^2 organelle	
	labelled	control	labelled	control	labelled	control
Cell wall	1	0	1.48	2.04	0.68	0
Cortical cytoplasm[1]	131	0	1.45	0.61	90.34	0
Central cytoplasm	168	2	9.95	14.85	16.88	0.13
Vacuoles	1	0	0.62	0.80	1.61	0
Plastids/mitochondria[2]	3	0	0.70	0.95	4.29	0
Nucleoplasm	59	1	8.53	7.85	6.92	0.13
Nucleolus	9	0	0.92	1.76	9.78	0

[1] Defined as a 70 nm thick layer of cytoplasm adjacent to the cell membrane.
[2] These organelles are scored together because of the difficulty to recognize them individually.

e.g., TOKUYASU 1980). It has to be noted, however, that, in spite of these advantages, interpretation of the distribution of label remains difficult. This is caused by so-called endogenous variations in penetration depth of the immunoreagents, due to difference of matrix density in various cellular compartments. In addition, exogenous factors, e.g. conditions of fixation and immunoreactions, can disturb the quantification procedure (SLOT et al. 1986).

Keeping these limitations in mind, we can nevertheless put forward that the label distribution, as shown in *table 1*, reflects the arrangement of tubulin in the various cellular organelles. An argument for this is that the dense labelling, present in the cortical regions of the cytoplasm (*fig. 2a*, arrows) agrees with the results obtained by immunofluorescence and ultrathin Epon sectioning (*figs. 1a, b, c*). In cell walls and vacuoles gold particles were found rarely and the labelling observed is regarded to be aspecific. It might either be caused by aspecific binding of the first antibody to nontubular cell components or it might be brought about by the attachment of the antibody to tubulin which was displaced due to cutting or stretching of the frozen sections. The gold labelling of plastids and mitochondria, which was scored together because of the difficulty to distinguish these organelles in the ultrathin cryosections, is higher; therefore, it cannot be excluded that some tubulin is present here. This view is supported by the observation of microtubule-like structures in the plastids of spinach (LAWRENCE & POSSINGHAM 1985). In the nuclei, and especially in the nucleoli of the embryonic cells, the immuno-gold labelling indicates the presence of tubulin. This was also registrated with light microscopy as a faint immunofluorescence in both the nucleus and the nucleolus. Also, in *Gasteria verrucosa* pollen a diffuse fluorescence was observed in the vegetative nucleus after tubulin labelling (VAN LAMMEREN et al. 1985). Microtubules were never observed directly in nuclei of plant cells; however, the tubulin nature of cristalloids in the nuclei

from leaves and stylar tissue of *Linaria vulgaris* has been suggested (CIAMPOLINI et al. 1980).

Although these observations are not yet conclusive and have to be chequed by immuno-blotting to exclude aspecific labelling, this paper indicates that immuno-gold labelling on ultrathin frozen sections might be used to localize tubulin in plant cells. It was, however, not yet possible to discern between the presence of intact MTs and tubulin subunits. The method will be applied to analyze the tubulin distribution during the various stages of somatic embryo development.

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