An Improved Immunolabeling Method for Microtubular Cytoskeleton in Poplar (*Populus nigra* L.) Free Nuclear Endosperm

Xu XuHan, André Souvré, Christel Granier and Michel Petitprez

Laboratory of Plant Breeding and Biotechnology, UA INPT/INRA, INP-ENSAT, 145 Av. de Muret, 31076 Toulouse Cedex, France

ABSTRACT. We investigated immunocytochemical staining of microtubular cytoskeleton of free nuclear endosperm, a tissue which is particularly difficult to fix. This tissue requires fixation for 45 hr to preserve the integrity of the microtubular network after paraformaldehyde based fixation. Low glutaraldehyde concentration in the fixative and the ethanol dehydration retains β-tubulin antigenicity and the former improves preservation of tissue structure. An ethanol-free embedding method is recommended for immunocytochemical studies of ethanol sensitive target proteins.

Key words: embryo sac, endosperm, fixation, immunolabeling, microtubule, *Populus nigra*

For decades, an important subject in biotechnology has been the search for reliable and easy microscopic methods to reveal cytological and histological events (Curr 1956, Stoward 1973, Juniper and Lawton 1979, Larsson 1988, Marc and Hackett 1989, Bell et al. 1989, Baskin et al. 1992, Kamo and Griesbach 1993, Dannenhoffer and Shen-Miller 1993). Large cytoplasm-rich or highly vacuolated tissues are among the most difficult materials for preserving morphology for histological and cytological research. Immunocytochemical staining is more complex because one must consider both the postfixation changes and the antigenicity of target proteins (De Mey et al. 1982, Traas et al. 1987, Sonobe and Shiboaoka 1989, Harold and Harold 1992, Larsson 1988). In plant tissue, the endosperm exhibits all of the troublesome characteristics. In most cases, the endosperm is a delicate tissue surrounding an extremely large central vacuole of the formal central cell. At early developmental stages, this tissue is a "liquid" coenocyte enveloped by a poorly permeable embryo sac wall in *Populus* and in other Angiosperms belonging to the Free Nuclear Type endosperm class (Li et al. 1982, XuHan and Van Lammeren 1993; for review see Vijayaraghavan and Prabhaker 1984). Consequently, it is difficult to use immunocytochemical methods to visualize cytological events in this tissue, especially at the whole tissue level, which may account for the limited amount of work that has been done with immunolabeling the microtubular cytoskeleton in the coenocytic endosperm (Van Lammeren 1988, Webb and Gunning 1990, XuHan and Van Lammeren 1993, 1994, Brown et al. 1994).

We have tested the effects of three parameters on the preservation of β-tubulin antigenicity and tissue structure: glutaraldehyde in paraformaldehyde-based fixative, duration of fixation, and omission of ethanol in sample processing. To achieve the last aim, an ethanol-free embedding method was used.

MATERIALS AND METHODS

Developing ovaries were collected at 20 to 30 days after anthesis from naturally grown *Populus nigra* plants. Nucelli were manually isolated by peeling off the integument tissue in the fixatives. In some cases, the endosperm was further isolated manually in the fixatives.

For immunocytochemical staining of microtubules (MTs), samples were fixed in 4% paraformaldehyde (w/v) in 0.1 M phosphate buffered saline (PBS tablet, Sigma Chemical Co. France), pH 7.0, and 0.1% Triton X-100 (w/v), or in the same fixative solution containing 0.1%

Address correspondence to André Souvré, Laboratory of Plant Breeding and Biotechnology, 145 Ave. de Muret, 31076 Toulouse Cedex, France
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glutaraldehyde (v/v) or 0.5% glutaraldehyde. Fixation periods of 3, 11, 24, and 45 hr at room temperature were tested.

Samples were embedded using either of two methods. In the first method, samples were dehydrated in serial ethanols of 30, 50, 70, 85, 95 and 100% (v/v) for 30 min each at room temperature. The samples were then transferred to a mixture of polyethylene glycol 1:3 (w/w) 4000 and 1500 (PEG4000, PEG1500, Merck-Schuchardt, Germany) dissolved 3:1, 1:1, and 1:3 (v/v) in ethanol at 50 C for 50 min each. The samples were then infiltrated three times in the PEG mixture without ethanol at 57 C for 30–60 min each, and solidified in the PEG mixture at room temperature. In the second method, samples were dehydrated in serial PEG2000 (Aldrich-Chemie S. à. r. l., France) solutions of 30, 50 and 70% (w/w) in PBS, each for 1–3 hr or overnight at room temperature, then three times in 100% PEG2000 or PEG3000 at 57 C for 30–60 min each. Samples were allowed to solidify in the PEG at room temperature. Heating is necessary to dissolve or melt PEG.

The embedded samples used for the present work were stored in small plastic pots at room temperature for less than two weeks, although we did not find loss of antigenicity in samples stored for three months under these conditions (data not shown). All samples were sectioned at 1.5 μm with an LKB 2218 Historange microtome. Sections were mounted on poly-L-lysine coated slides (poly-L-lysine, Sigma, St. Louis, MO; 0.1% (w/v)) using a small loop with PEG1500 water solution in appropriate concentrations, adjusted by the user to permit the sections to stretch properly. After three washes in PBS for 10 min each, sections were treated with 5% normal goat serum (Amersham Int. plc, UK) for 0.5 hr at 30 C to reduce nonspecific labeling. Immunolabeling was performed at 35 C using a monoclonal anti-β-tubulin (Amersham Intl. plc, UK) as the first antibody at 1:500 dilution for 1 hr and fluorescein isothiocyanate (FITC) linked sheep anti-mouse IgG (Amersham Intl.) as the secondary antibody at 1:100 dilution for 1 hr. Three washes for 10 min each in PBS followed each labeling. Controls were made by omitting the first antibody. Sections were further stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Chimie, France) added to mounting Citifluor (Link Analytical, UK) at final concentration of 0.02% (w/v) to reveal nuclei and DNA-carrying organelles. Observations and photomicrographs were taken with a Leitz Laborlux 12 microscope with a Wild Photoautomat MPS45 exposure system using TMAX P3200 film.

RESULTS

Duration of Fixation

Fixation treatments for four periods were compared (Table 1). Fixation for 3 hr revealed no photo-recordable MT arrays in the coenocytic endosperm, except for occasional, short fluorescent lines. This was recorded, however, as "no labeling," although all the sections appeared dark brown (labeling silence) in the control omitting anti-β-tubulin.

Prolonged fixation resulted in increased fluorescence of the MT arrays stained by anti-β-tubulin and FITC linked secondary antibody. After 11 hr fixation, part of the MT network was visible at times. After 24 hr fixation, an incomplete MT network in the endosperm was observed. In the latter case, doubling the concentrations of both antibodies did not enhance labeling and increased noise. These results suggested testing a longer period of fixation. Therefore, tests using 45 hr fixation were carried out to compare the effects of 0.1% and 0.5% glutaraldehyde (Table 1).

After 45 hr fixation, a large MT network was visualized in the endosperm. In a few cases, the

Table 1. Effects of Duration of Fixation, Glutaraldehyde Concentration and Ethanol on Immunocytochemical Labeling of Microtubules and Tissue Structure Preservation

Fixation time (hr)	Glutaraldehyde Concentration in Fixatives (%)				
	0	0.1	0.5	0	0.5
	Normal Embedding			Ethanol-Free Embedding	
3	−	−	−	−	−
11	*	+	+	+	+
24	++	++	*	++	++#
45	*	+++#	*	*	+++##

−, no labeling; *, not determined; +, poor labeling; ++, unsatisfactory labeling; +++, good labeling; #, improved tissue structure preservation; ##, greatly improved tissue structure preservation.

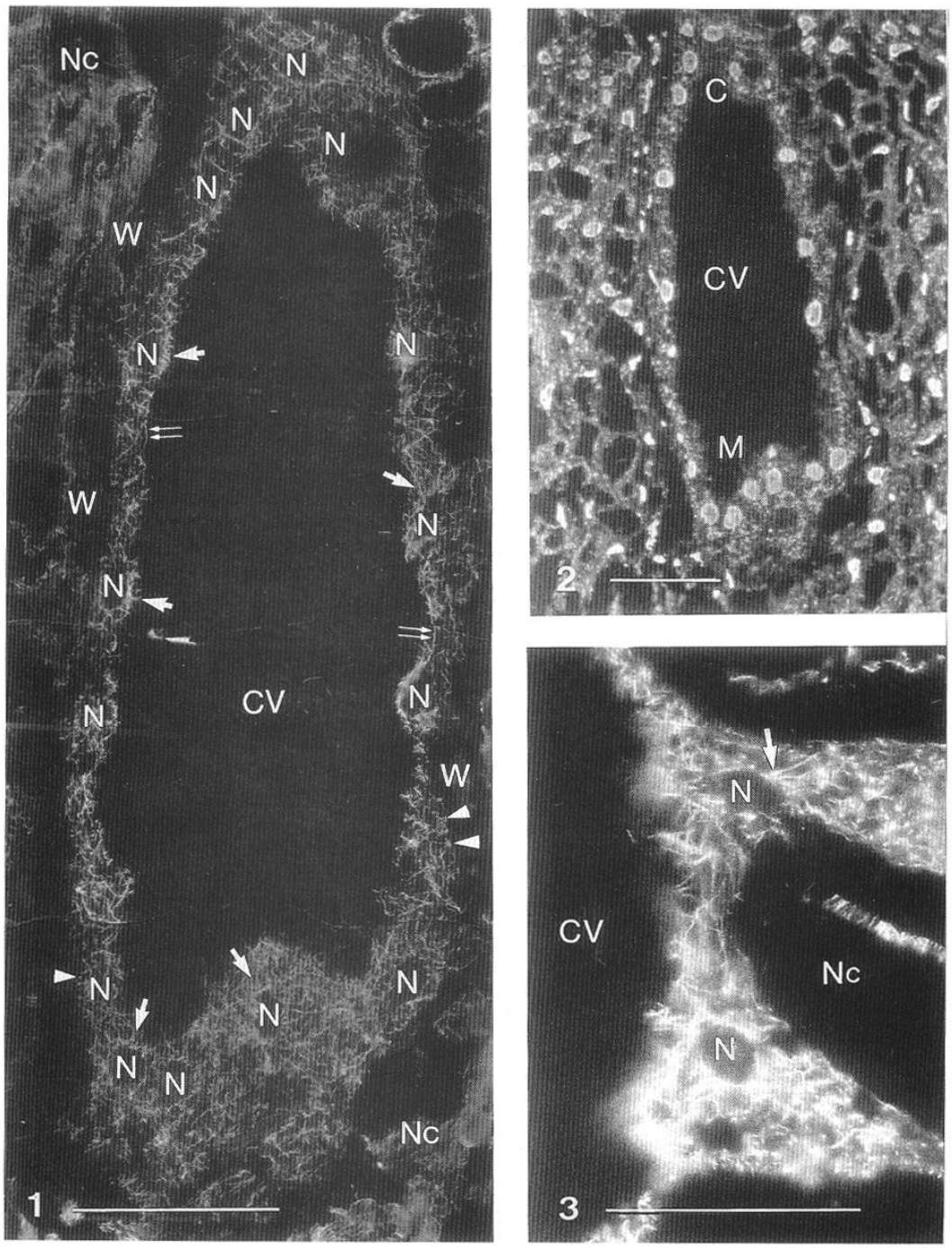

Fig. 1. Fluorescence micrograph of a longitudinal section of a poplar nuclear endosperm immunocytochemically stained with anti-β-tubulin. Note the three populations of MTs in the framework: radiating arrays (arrows) from the nuclear surfaces, cytoplasmic arrays (fat arrows) in the internuclear areas, and a few cortical arrays (arrowheads) near the cell wall of the embryo sac. Endosperm was fixed for 45 hr in 4% paraformaldehyde and 0.1% glutaraldehyde, and processed via the ethanol-mediated PEG procedure. V, central vacuole; N, nucleus of endosperm; NC, nucellus tissue; W, embryo sac wall. Bar = 50 μm.

Fig. 2. Fluorescence micrograph at a low magnification showing nuclear position in the same section as in Fig. 1 stained with DAPI. C, chalazal part; M, micropylar part; V, central vacuole of the formal central cell. Bar = 50 μm.

Fig. 3. Fluorescence micrograph of an oblique section of the embryo sac showing the radiating arrays of MTs and the orientations of the cytoplasmic MTs. Arrow indicates a possible site of MT nucleation. Endosperm was fixed for 45 hr in 4% paraformaldehyde and 0.1% glutaraldehyde, and processed via the ethanol-mediated PEG procedure. V, central vacuole; N, nucleus of endosperm; NC, nucellus tissue. Bar = 50 μm.

staining of the network was not observed over the whole endosperm, e.g., in the micropylar part where the embryo was present. As a rule, however, images of long and thick bundles and small and short arrays of MTs interweaving into an integral framework in the endosperm tissue were easily obtained (Fig. 1). By comparing with DAPI staining (Fig. 2), it was clear that the vacuoles and nuclei were not stained (Figs. 1 and 3). In the MT framework shown in Figs. 1 and 3, three populations of MTs were visualized: the perinuclear arrays of MTs including the arrays radiating from the surfaces of the nuclei, cytoplasmic arrays of MTs interwoven in the internuclear spaces constituting most of the MT network, and some cortical MTs close to the embryo sac wall. The MTs were more extensive where the nuclei were close to each other as in the micropylar or chalazal part. The outer tissues, such as the nucellus, sometimes exhibited loss of tubulin antigenicity after 45 hr fixation.

Effect of Glutaraldehyde

Glutaraldehyde neither decreased MT specific labeling nor increased nonspecific labeling in the concentrations tested here, nor did it significantly enhance preservation of MTs over that obtained by fixation without glutaraldehyde. Prolonged fixation also produced no obvious effects of glutaraldehyde on specific or nonspecific labeling under conditions tested. At the level of overall structure of sectioned tissue, however, we found that glutaraldehyde preserved the integrity of the endosperm and other tissues. This effect occurred with 0.5% glutaraldehyde fixation for 24 hr and ethanol-free embedding, and it was further enhanced by prolonged fixation for 45 hr. The same effect was obtained using the normal embedding procedure after 0.1% glutaraldehyde fixation for 45 hr (Table 1). Samples fixed with glutaraldehyde for 24 and 45 hr maintained tissue integrity when sections were mounted on slides. To avoid tissue compression, it was necessary to leave the section in the mounting PEG solution in the loop for several seconds or until the solution reached equilibrium before the section was affixed to the slide.

Ethanol-Mediated and Ethanol-Free Embedding

The use of ethanol for dehydration and transition to various types of PEG did not produce loss of MT labeling in the MT configuration compared to ethanol-free processing (Table 1).

We used a graded PEG-PBS solution series instead of ethanol-mediated transfer to embedding PEG. We found 2000 and 3000 molecular weight PEGs were suitable because the viscosity of 70% solution is low at room temperature. A regimen of 30, 50, and 70% PEG-PBS solutions at room temperature and 100% PEG at 57 C was adopted because samples did not show structural compression when the 80 and 90% PEG-PBS steps were omitted. When isolated young nucelli or endosperms were used, however, infiltration in PEG-PBS solutions led to compression of the tissue starting in 7% PEG solution. In general, the infiltration is slower than the ethanol-mediated regime.

Samples could stay overnight in the PEG solution at any step (before the warm PEG) without loss of antigenicity of β-tubulin. In 100% PEG, at least two changes were necessary to maintain the hardness of the embedded block for 1–3 μm sectioning at room temperature.

DISCUSSION

The greatest change over the years in fixation techniques (Curr 1956) lies in the modification from lethal to semi- or nonlethal fixation to preserve properties of proteins such as antigenicity for immunohistochemical studies. Proper in situ immunolabeling of MTs depends on optimizing various parameters during sample processing. Compared with previous work, the method presented here results in a clear image of the MT framework in the nuclear endosperm of sectioned whole tissues of Angiosperms.

In this work, we continue to use Triton to enhance fixative penetration, whereas to simplify the test of fixation parameters, we avoided using MT stabilizing buffer containing EGTA (ethylene glycol-bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid), PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid) (Traas et al. 1987, XuHan and Van Lammeren 1993), and aldehyde reducing agents such as sodium cyanoborohydride ($NaBH_3CN$) (Barney et al. 1990, Mattson et al. 1990). Although these chemicals may be important for immunolabeling MTs, the method presented here yields clear images of MT arrays that are sharper than those obtained from the small coenocytic endosperm of *Ranunculus sceleratus* (XuHan and Van Lammeren 1993). Because ethanol and a long fixation time in glutaraldehyde were thought to be responsible for loss of antigenicity, earlier studies used cryosectioning, and avoided ethanol and glutaralde-

hyde. The present study, however, indicates that neither ethanol dehydration and transition to embedding medium nor the glutaraldehyde-containing fixatives adversely affects antigenicity of β-tubulin in the endosperm.

The ethanol-free procedure reported in this paper does not enhance visualization of MTs compared to the ethanol-mediated method. It can, however, be used in cases where target proteins are ethanol sensitive and the timing of the processing is less critical.

The mechanisms of aldehyde function in fixatives are discussed by Jones (1973) and Hopwood (1973). The sharp image of MTs in our work is due to the prolonged fixation that preserves both the structure of the tissue and the antigenicity of the β-tubulin. The antigenicity of β-tubulin may be preserved during the long fixation time due to limited quantities of aldehydes penetrating the tissue, and any that do penetrate may be bound competitively by other elements in the tissue. We estimate that the maximal fixation time is close to 45 hr because the surrounding nucellar tissue starts to lose β-tubulin antigenicity at this time, suggesting that antigenicity would soon be lost in the endosperm as well.

In samples fixed for 45 hr in glutaraldehyde-containing fixatives, we observed that the cytoplasmic MTs run throughout the cytoplasm of the poplar nuclear endosperm. Although interconnecting with the radiating and cortical MTs, they form an independent population. Little attention has been given to the cytoplasmic population, which has not been properly classified as have other kinds of microtubular organizations in plant cells (Goddard et al. 1994). The results reported here indicate that the cytoplasmic population of MTs may be an independent type of organization analogous to animal cells (Ingber et al. 1994). This organization stands next to the radiating arrays, the preprophase band, the cortical arrays, the spindle, and the phragmoplast organizations. Poor fixation may have caused investigators to overlook the phenomenon. The cytoplasmic MTs were also noticed by Baskin et al (1992) in plant cells after an improved methacrylate embedding method, further suggesting the importance of improving immunocytochemical techniques.

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