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Actin Filaments and Microtubules in the Preprophase Band and Phragmoplast of Tobacco Cells

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Summary

Treatment with lysine prior to fixation of tobacco BY-2 cells with formaldehyde improved the preservation of actin filaments in the cells and enabled us to observe both networks of actin filaments and microtubules in the same cells. By using this method, we observed that (1) actin filaments were present in the preprophase band; (2) the actin filaments in the preprophase band and phragmoplast were runnig in the same direction as the microtubules in their respective structures; (3) a cortical network of actin filaments was present throughout all stages of cell cycle.

The present method did not preserve the cortical actin filaments in interphase cells. The procedure for staining microtubules destroyed them.

Keywords: Actin filaments, Microtubules, Phragmoplast, Preprophase band, Tobacco BY-2 cell.

Abbreviations: EGTA Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PIPES Piperazine-N,N′-bis(2-ethanesulfonic acid); PMSF Phenylmethylsulfonyl fluoride; TLCK Na-p-tosyl-L-lysine chloromethyl ketone.

1. Introduction

The use of fluorescent dye-labeled phallotoxins (PESACRETA *et al.* 1982) has enabled us to visualize actin filaments in plant cells. Observations using this technique have revealed the three dimensional architecture of the filaments in cells of a wide variety of plants (PARTHASARATHY *et al.* 1985, CLAYTON and LLOYD 1985, TIWARI *et al.* 1984). The phallotoxin staining has often been used in combination with indirect immunofluorescent staining for microtubules to clarify the spatial relationships between networks of actin filaments and microtubules. By this combined double staining method, CLAYTON and LLOYD (1985) revealed that actin filaments are present in the phragmoplast of onion root-tip cells.

The double staining technique, using fluorescent-phallotoxins and anti-tubulin antibodies, is useful for studying cytoskeletal networks in plant cells, but the preservation of both actin filaments and microtubules in the same cells continues to present difficulties.

Actin filaments are known to be sensitive to fixatives. It has been reported that better preservation of the filaments can be achieved by a brief fixation with low concentrations of paraformaldehyde than by prolonged fixation with higher concentrations of the aldehyde (PARTHASARATHY *et al.* 1985). PIERSON *et al.* (1986) reported that simultaneous treatment with fixative and fluorescein-labeled phalloidin greatly enhanced the preservation of actin filaments in lily pollen tube cells.

A brief fixation with low concentrations of paraformaldehyde permits good preservation of actin filaments, but such fixation does not preserve microtubules. Furthermore, prolonged fixation with high concentrations of paraformaldehyde, which is sufficient for the preservation of microtubules, is too strong to preserve actin filaments. Thus, to observe both actin filaments and microtubules in the same cells, we must devise an appropriate fixation method that preserves microtubules without destroying actin filaments.

Recently, BOYLES *et al.* (1985 a, b) reported that the

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addition of lysine to the aldehyde fixative greatly improved the preservation of actin filaments assembled *in vitro* and the preservation of actin filaments in platelets.

We have used lysine for fixation of plant cells and succeeded in preserving microtubules without destroying actin filaments. We have been able to visualize both actin filaments and microtubules in the same cells by staining with rhodamine-labeled phalloidin and fluorescein-labeled anti-tubulin antibody.

In the present paper, we describe the details of the newly devised method and report the results of observations obtained by this method.

2. Materials and Methods

2.1. Plant Material

BY-2 cells of tobacco (*Nicotiana tabacum* "Bright Yellow 2") were cultured in a suspension in modified Linsmaier and Skoog's medium, supplemented with 3% sucrose and 0.2 mg/ml 2,4-dichlorophenoxyacetic acid, pH 5.8, at 26 °C in the dark. The cells were subcultured every 7 days (NAGATA *et al.* 1981).

2.2. Staining for Actin Filaments

Actin filaments in the cells were stained by the following two procedures: 1. The cells were extracted with lysis buffer (100 mM PIPES buffer, pH 7.0, which contained 0.1% Triton X-100, 1 mM $MgCl_2$, 5 mM EGTA, 3 mM dithiothreitol, 0.3 mM PMSF, 50 μg/ml leupeptin, 20 μM taxol and 0.1% n-propylgallate) for 20 minutes at 35 °C. Extracted cells were stained by mixing a suspension of cells in lysis buffer with the same volume of rhodamine-phalloidin solution (0.66 μM rhodamine-labeled phalloidin in phosphate buffered saline (PBS: 10 mM sodium phosphate buffer, pH 7.3, containing 0.8% NaCl) which contained 1% bovine serum albumin). 2. The cells were incubated in a mixture of lysis buffer and rhodamine-phalloidin solution (1 : 1 v/v) for 10 minutes at 35 °C.

As we could not distinguish between the arrangements of actin filaments visualized by these two procedures, the second procedure was employed when the cells were subsequently stained with anti-tubulin antibody.

2.3. Staining for Microtubules

Microtubules in the cells were visualized by indirect immunofluorescence staining by the procedures described by WICK *et al.* (1981). After confirming that actin filaments in all cells were stained, an equal volume of KP-EGTA buffer (50 mM potassium phosphate buffer, pH 7.0, containing 5 mM EGTA and 0.3 mM PMSF) which contained 1.5% lysine was added to the suspension of cells in a mixture of lysis buffer and rhodamine-phalloidin solution. The resultant mixture was kept at 4 °C for 1 hour and then mixed with the same volume of KP-EGTA buffer which contained 6.2% formaldehyde, and kept at room temperature for 40 minutes. After washing with KP-EGTA buffer, the cells were incubated with enzyme solution (aqueous solution of 0.8% Cellulase "Onozuka" RS, 0.08% Pectolyase Y 23, 5 mM EGTA, 50 μg/ml leupeptin, 10 μg/ml TLCK, 10 μg/ml pepstatin A, and 0.3 mM PMSF, pH 5.8) for 10 minutes at 26 °C to digest cell walls. The cells were washed with KP-EGTA buffer, and were incubated with mouse monoclonal anti-chick brain α-tubulin diluted 1 : 500 in PBS, which contained 1% bovine serum albumin and 0.1 μM rhodamine-phalloidin, at room temperature for 45 minutes. The cells were washed with PBS which contained 0.05% Tween-20 for 5 minutes, and then incubated with fluorescein-labeled rabbit anti-mouse IgG diluted 1 : 10 in PBS which contained 1% bovine serum albumin and 0.1 μM rhodamine-phalloidin. The stained cells were washed with PBS which contained Tween-20 for 5 minutes and mounted with Gelvatol (RODRIGUES and DEINHARDT 1960) which contained 0.1% paraphenylenediamine.

2.4. Microscopy

The cells were examined with a microscope with epifluorescence illumination (Olympus BHS-RFK, Olympus Tokyo, Japan).

Actin filaments stained with rhodamine were examined by using BP 545 and EO 530 as exitation filters and O 590 as a barrior filter. Microtubules stained with fluorescein were examined with BP 490 and EY 455 as excitation filters and O 515 as a barrior filter. Use of these combinations of filters completely discriminated between the signals from these two different dyes.

2.5. Antibodies and Enzymes

Monoclonal anti-chick brain α-tubulin antibody was purchased from Amersham (Buckinghamshire, England, UK) and fluorescein-labeled rabbit anti-mouse IgG from Miles Scientific (North Aurora Road, Naperville, Israel). Cellulase "Onozuka" RS was obtained from Yakult Honsha Co., Ltd. (Tokyo, Japan) and Pectolyase Y 23 was from Seishin Pharmaceutical Co., Ltd. (Tokyo, Japan).

3. Results and Discussion

3.1 Actin Filaments and Microtubules in Preprophase

Treatment with lysine prior to fixation with formaldehyde made possible the preservation of microtubules without the destruction of actin filaments. We were able to observe the networks of actin filaments and microtubules in the cells stained by the method described above (Figs. 1 and 2).

As mentioned in Materials and Methods, the arrangements of actin filaments visualized by the procedures in which cells were extracted with lysis buffer containing no phalloidin first and then stained with rhodamine-phalloidine were almost the same with those visualized by the present method. These results may allow us to consider that the patterns of staining with rhodamine-phalloidin shown in the present paper represent the arrangements of actin filaments *in situ*, although we can not exclude the possibility that the patterns of staining are artifacts produced by phalloidin in lysis buffer. The arrangements of microtubules visualized by the present method were almost identical with those visualized by the ordinary method in which fixed cells were stained. Probably, taxol in lysis buffer caused little or no artifacts.

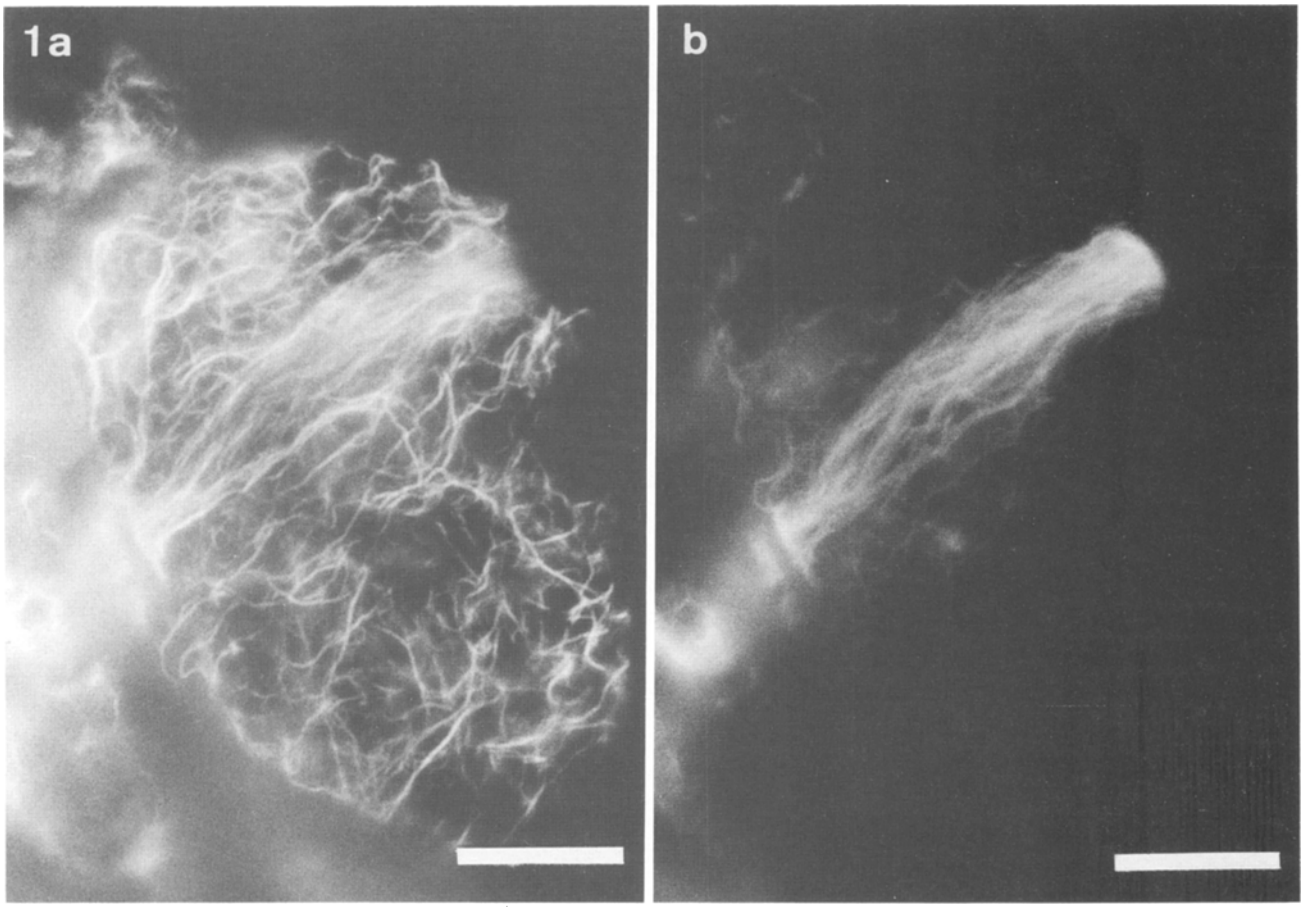

Fig. 1. Actin filaments and microtubules in a single preprophase cell. *a* Actin filaments. *b* Microtubules. Bars equal 10 μm

Fig. 1 shows networks of actin filaments and microtubules in a cell in preprophase. Microtubules in the preprophase band were well preserved (Fig. 1 *b*). In preprophase cells, actin filaments could be detected both at the position of the preprophase band and over the entire area of the cell cortex (Fig. 1 *a*). Actin filaments at the position of the preprophase band were found to be running in the same direction as the microtubules in the band and the actin filaments which spread over the entire area of cell cortex to form a meshwork. To our knowledge, the presence of actin filaments in the preprophase band has not previously been demonstrated. We think that the improvements in the staining method have enabled us to demonstrate this phenomenon for the first time. Actin filaments in the preprophase band disappeared when microtubules in the band disappeared, but the meshwork of actin filaments which spread over the entire area of the cell cortex remained and was present throughout all stages of cell division (see Fig. 2 *a*).

As long as the preprophase band region is concerned, the pattern of staining for actin filaments bears close resemblance to that for microtubules (Fig. 1), suggesting strong interactions between these two cytoskeletal elements. Possibly, these interactions help to stabilize both actin filaments and microtubules in the preprophase band.

3.2. Actin Filaments and Microtubules in Telophase

Fig. 2 shows actin filaments and microtubules in telophase. As mentioned above, the meshwork of actin filaments near the cell cortex was still present in telophase cells (Fig. 2 *a*). Besides being located near the cell cortex, actin filaments were present in the phragmoplast (Fig. 2 *b*). As has been shown in onion root-tip cells (CLAYTON and LLOYD 1985), *Haemanthus* endosperm cells (SCHMIT and LAMBERT 1985, SCHMIT *et al.* 1985) and *Tradescantia* stamen hair cells (GUNNING and WICK 1985), actin filaments were co-distributed with

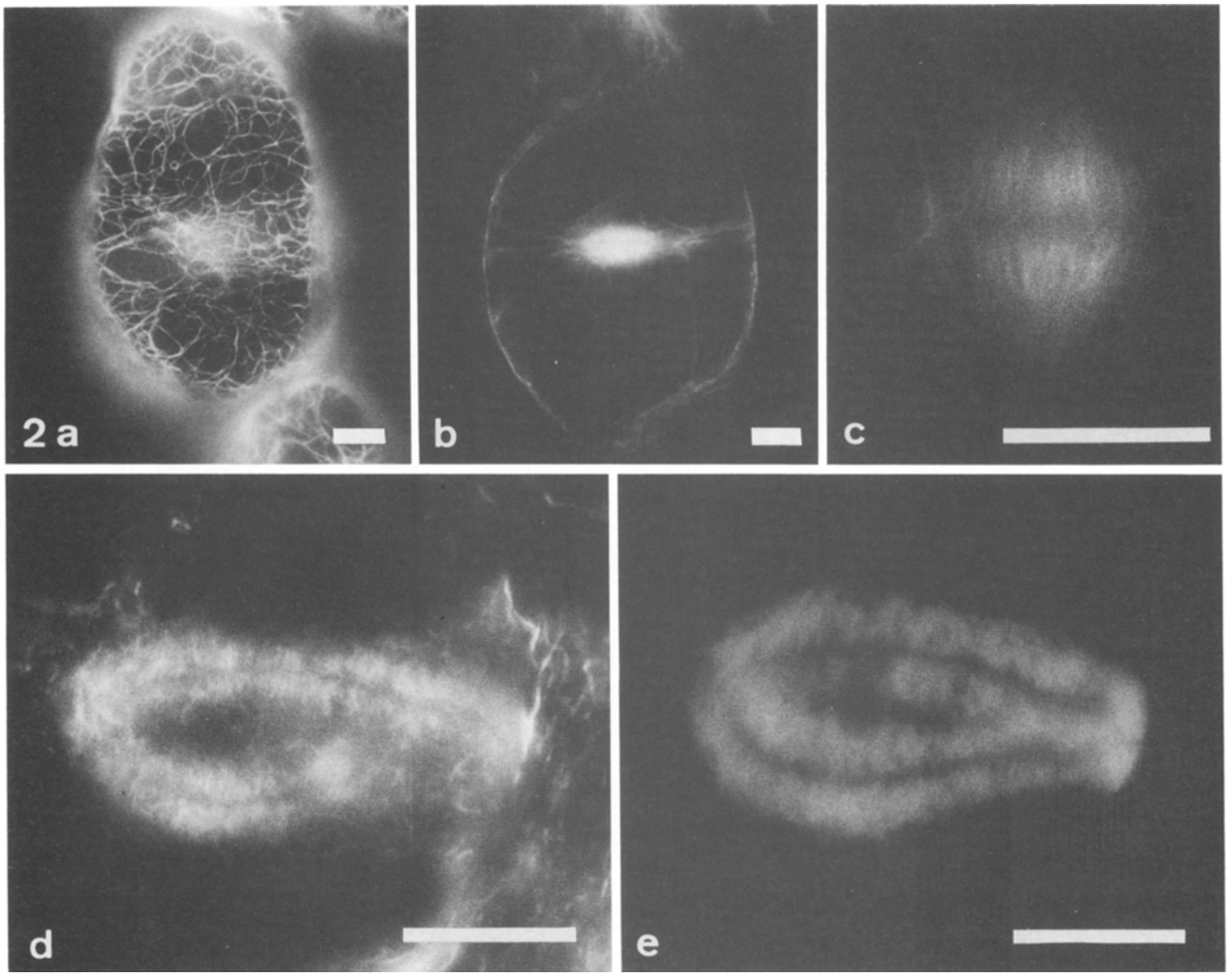

Fig. 2. Actin filaments and microtubules in telophase cells. *a* and *b* Actin filaments in a single cell. *a* At the level of the cell surface. *b* At the level of the phragmoplast. *c* Actin filaments in a phragmoplast in its early stage. *d* and *e* Actin filaments and microtubules in a single phragmoplast in its late stage. *d* Actin filaments. *e* Microtubules. Bars equal 10 μm

microtubules in the phragmoplast of tobacco BY-2 cells (Figs. 2 *d, e*). The resolution of the present method seems to be better than that of the methods described by the previous workers. The photographs of the actin filaments in the phragmoplast in their papers do not allow us to speculate about the arrangement of the filaments. However, our result (Figs. 2 *c, d*) strongly suggests that actin filaments in the phragmoplast are running parallel to the internuclear axis in the same way as the phragmoplast microtubules. This arrangement of actin filaments strongly suggests the involvement of actin filaments in transport of material into the cell plate.

Actin filaments can be broken into short fragments by treatment with glutaraldehyde (LEHRER 1981). Thus, it may be possible that the high concentrations of paraformaldehyde, which the previous workers used in their studies, caused fragmentation of actin filaments in the phragmoplast and thereby obscured the arrangement of the filaments.

Fig. 2 *b* shows the presence of actin filaments lying between the cell cortex and the phragmoplast, which was not demonstrated in *Tradescantia* stamen hair cells (GUNNING and WICK 1985). Actin filaments seem to be involved also in the spatial control of cytokinesis in tobacco BY-2 cells.

Unlike in *Tradescantia* stamen hair cells where actin filaments were distributed uniformly across the phragmoplast even in late stages of cytokinesis (GUNNING and WICK 1985), in tobacco BY-2 cells actin filaments were not present in the central region of the late phragmoplast (Fig. 2 *d*).

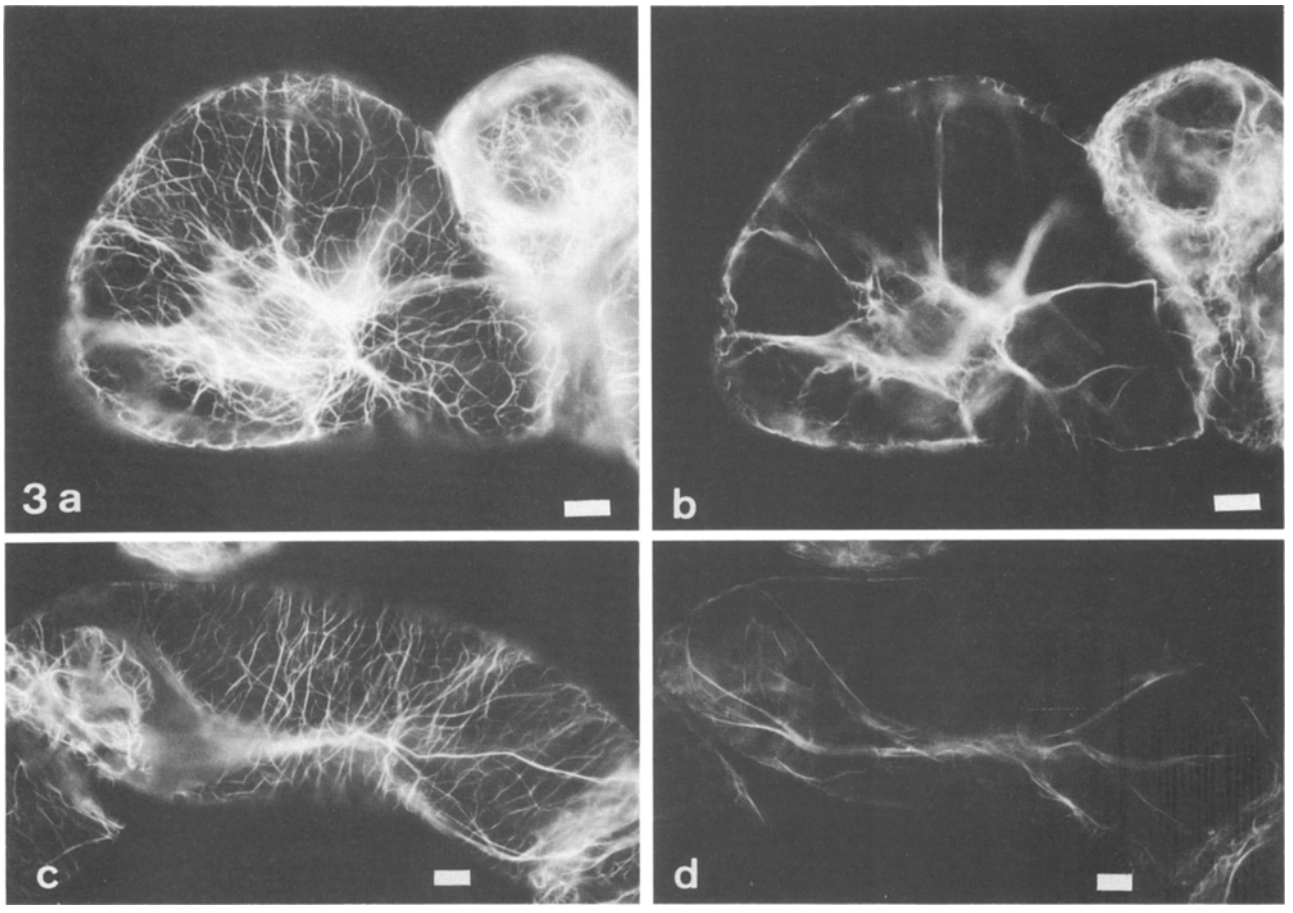

Fig. 3. Actin filaments in interphase cells. *a* and *b* Two focal planes of a cell. *a* At the level of the cell surface. *b* At the level of the nucleus. *c* and *d* Two focal planes of an elongated cell. *c* At the level of the cell surface. *d* At the level of the nucleus. Bars equal 10 μm

SCHMIT and LAMBERT (1985) reported that the ring of actin filaments appeared at the equatorial region of *Haemanthus* endosperm cells in mid-anaphase when the phragmoplast had not yet appeared. In tobacco BY-2 cells, such a ring of actin filaments was not observed before the appearance of the phragmoplast. In *Haemanthus* endosperm cells, it was also reported that actin filaments in the telophase phragmoplast were distributed only near its midline (SCHMIT and LAMBERT 1985). But, in tobacco BY-2 cells the phragmoplast actin filaments occupied rather broad regions. They expanded toward the daughter nuclei as the phragmoplast microtubules, although the former seemed slightly shorter than the latter (Figs. 2 *d, e*).

3.3. Actin Filaments in Interphase

Staining with rhodamine-phalloidin revealed that actin filaments are present near the cell cortex as well as in cytoplasmic strand in tobacco BY-2 cells (Fig. 3), as has been reported for wheat coleoptile cells by PARTHASARATHY (1985). Transversely oriented actin filaments are predominant in the cell cortex of elongated cells (Fig. 3 *c*). The double staining did not give a satisfactory result. The procedure for staining microtubules destroyed the cortical actin filaments. Our method needs to be improved to allow staining of both cortical actin filaments and cortical microtubules in the same interphase cell, so that we can determine how they are spatially interrelated.

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References

BOYLES J, ANDERSON L, HUTCHERSON P (1985 a) A new fixative for the preservation of actin filaments: Fixation of pure actin filament pellets. J Histochem Cytochem 33: 1116–1128

BOYLES J, FOX JEB, PHILLIPS DR, STENBERG PE (1985b) Organization of the cytoskeleton in resting, discoid platelets: Preservation of actin filaments by modified fixation that prevents osmium damage. J Cell Biol 101: 1463–1472

GUNNING BSE, WICK SM (1985) Preprophase bands, phragmoplasts, and spatial control of cytokinesis. J Cell Sci (Suppl) 2: 157–179

CLAYTON L, LLOYD CW (1985) Actin organization during the cell cycle in meristematic plant cells. Exp Cell Res 156: 231–238

LEHRER SS (1981) Damage to actin filaments by glutaraldehyde: Protection by tropomyosin. J Cell Biol 90: 459–466

NAGATA T, OKADA K, TAKEBE I, MITSUI C (1981) Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). Mol Gen Genet 184: 161–165

PARTHASARATHY MV (1985) F-actin architecture in coleoptile epidermal cells. Eur J Cell Biol 39: 1–12

— PERDUE TD, WITZTUM A, ALVERNAZ J (1985) Actin network as a normal component of the cytoskeleton in many vascular plant cells. Am J Bot 72: 1318–1323

PESACRETA TC, CARLEY WW, WEBB WW, PARTHASARATHY MV (1982) F-actin in conifer roots. Proc Natl Acad Sci USA 79: 2898–2901

PIERSON ES, DERKSON J, TRAAS JA (1986) Organization of microfilaments and microtubules in pollen tubes grown *in vitro* or *in vivo* in various angiosperms. Eur J Cell Biol 41: 14–18

RODRIGUES J, DEINHARDT F (1960) Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 12: 316–317

SCHMIT AC, LAMBERT AM (1985) F-actin dynamics is associated to microtubule function during cytokinesis in higher plants. A revised concept. In: DE BRABANDER, DE MAY J (eds) Microtubules and microtubule inhibitors 1985. Elsevier Science Publishers, Amsterdam, pp 243–252

— VENTARD M, LAMBERT AM (1985) Microtubule and F-actin rearrangement during the initiation of mitosis in acentriolar higher plant cells. In: ISHIKAWA H, HATANO S, SATO H (eds) Cell motility: Mechanism and regulation. Univ Tokyo Press, Tokyo, pp 415–433

TIWARI SC, WICK SM, WILLIAMSON RE, GUNNING BES (1984) Cytoskeleton and integration of cellular function in cells of higher plants. J Cell Biol 99: 63s–69s

WICK SM, SEAGULL RW, OSBORN M, WEBER K, GUNNING BES (1981) Immunofluorescence microscopy of organized microtubule array in structually stabilized meristematic plant cells. J Cell Biol 89: 685–690