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Actin distribution in mitotic apparatus of somatic embryo cells of Norway spruce

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Abstract

F-actin distribution was studied in mitotic cells of embryogenic suspension culture of Norway spruce [*Picea abies* (L.) Karst.]. Actin was present in dividing cells of embryo head during whole mitosis. Transient co-localization of actin microfilaments with preprophase band of microtubules was observed. Weak actin staining occurred with non-kinetochor microtubular fibers in metaphase spindle. F-actin was not localized with kinetochore microtubular fibres in metaphase as well as with shortening kinetochore fibres in late anaphase. On the other hand, abundant actin microfilaments array was formed in the area of late anaphase spindle in equatorial level of the cell between separating chromatids. F-actin was also present in phragmoplast area in telophase.

Additional key words: microtubule, microfilament, mitosis, phragmoplast, *Picea abies*, preprophase band, tubulin.

Introduction

Early somatic embryo in Norway spruce is organized as a longitudinal structure that consists of cells of embryonal group, embryonal tubes and embryonal suspensor. The cells of embryonal group are isodiametric and easy to distinguish from the rest of the somatic embryo. While most of the cells of embryonal tubes and suspensor loose their capability to divide, embryonal cells are rapidly dividing. Embryonal cells provide an excellent experimental system for study of the cytoskeleton rearrangement during the cell cycle progression.

Tubulin, a major cytoskeletal component, plays an important role in different cellular events including formation of the mitotic apparatus. Microtubular cytoskeleton was studied in conifers by Hakmann *et al.* (1987), Tautorus *et al.* (1992), Fowke *et al.* (1995), Binarová *et al.* (1996) and its role in mitotic apparatus organization was described.

Actin, another major cytoskeletal component is involved in various cellular processes, such as motility, cytoplasmic streaming, chromosome segregation, cytokinesis and control of the cell shape including cell elongation (Schwob *et al.* 1992, Thimann *et al.* 1992, Ingber *et al.* 1995, Blancaflor *et al.* 1997). Actin and tubulin are detected in significant amounts in the cytoplasm and both cytoskeletal proteins are detected also in nuclei (Armbruster *et al.* 1983, Amankwah *et al.* 1994). Perinuclear F-actin shells were generally present in all eukaryots (Clubb *et al.* 1996). Actin filaments were recognized as widespread components in plant cells (Lloyd 1988), but data about the role of actin during mitosis in plant cells are controversial. Mitotic actin structures together with microtubules play the role in chromosome movement and also were linked with the cell plate formation (Czaban *et al.* 1993, Forer *et al.* 1994). On

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Abbreviations: DAPI - 4,6-diamidino-2-phenylindole; MTSB - microtubule stabilizing buffer; PBS - buffer saline; PPB - preprophase band
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the other hand, the absence of actin microfilaments in mitotic spindle was often reported (Cho *et al.* 1991, McCurdy *et al.* 1990).

Here we describe F-actin distribution during cell cycle progression in conifer cells with the aim to elucidate its distribution and its possible role in mitotic apparatus organization.

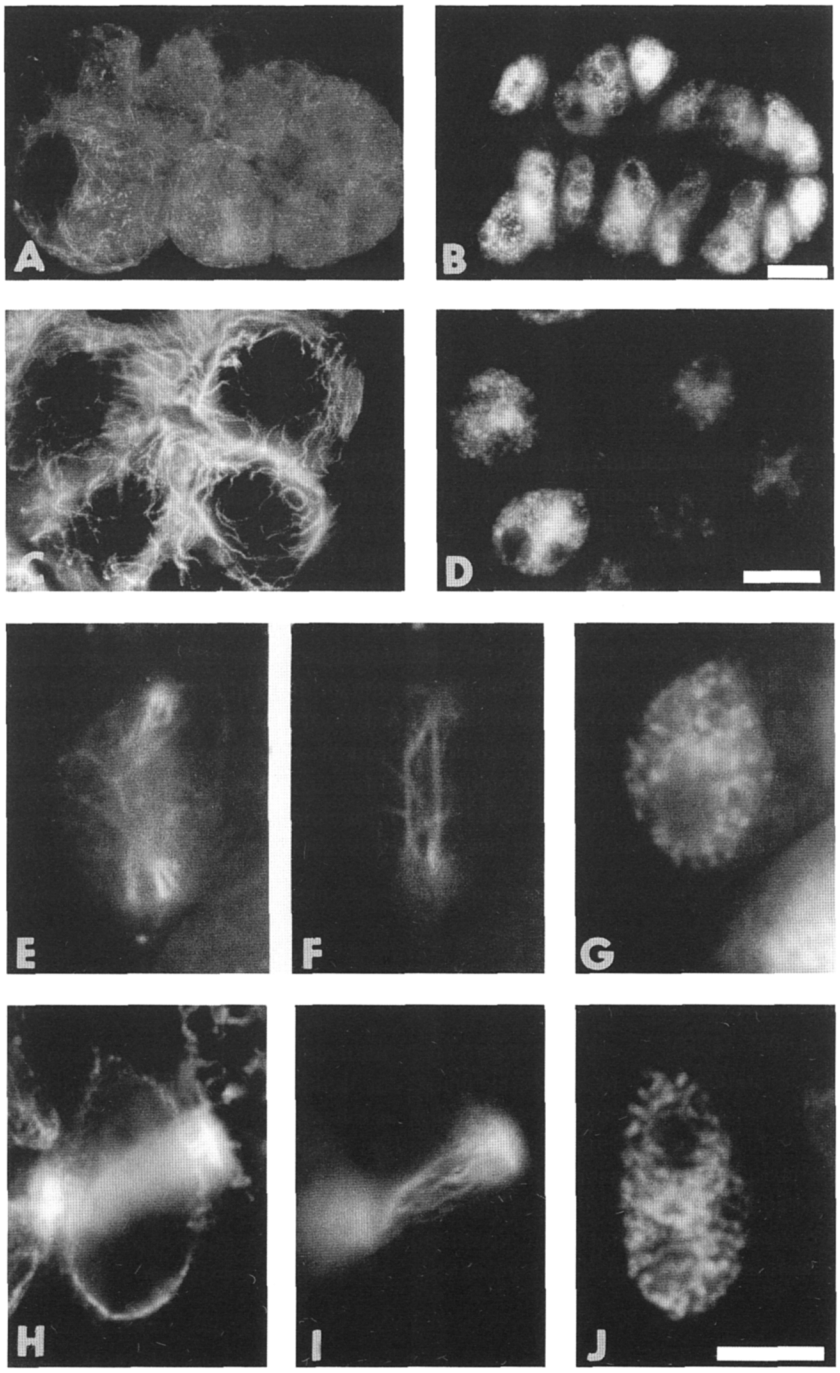

Fig. 1. Somatic embryogenic cells of spruce - interphase, prophase. Actin staining with rhodamine phalloidine (*A,E,F*), tubulin staining with FITC conjugated antibody (*C,H,I*), DAPI staining of chromatin (*B,D,G,J*). A very fine meshwork of randomly oriented actin microfilaments in small rapidly dividing embryonal cells with big nuclei and dense cytoplasm (*A,B*); randomly oriented cortical and cytoplasmic microtubules form extensive network in embryo head cells (*C,D*); cortical and subcortical band of actin microfilaments located in equatorial plane of preprophase cell form actin PPB (*E,G*), which occurred not only in cortex, but deeper in cytoplasm (*F*); microtubular PPB as a regular part of preprophase cells (*H,J*); microtubular PPB and perinuclear fluorescence of tubulin (*H*); fine meshwork of microtubules around nuclear envelope in preprophase cell (*I*). *Bars* = 10 μm.

Materials and methods

Early somatic embryos of Norway spruce [*Picea abies* (L.) Karst.] induced from ripe zygotic embryos (Havel *et al.* 1992) were used in experiments. The suspension culture of early somatic embryos was established from clone E0 in liquid half-strength LP medium (von Arnold 1987) with 15 mM NH_4NO_3, 3 mM L-glutamin, 9 μM 2,4-dichlorophenoxyacetic acid, and 4.4 μM N^6-benzyl-adenine (Durzan *et al.* 1994). Cultures were maintained stationary in 5 cm^3 liquid medium in Erlenmayer tubes (25 cm^3) in darkness at 24 °C. Suspensions were subcultured every 7 d.

Immunofluorescence of actin and tubulin: Actin microfilaments in unfixed cells were stained with rhodamin-phalloidin as described by Binarová *et al.* (1996). Shortly, suspension cultures of early somatic embryos were incubated in dark in staining solution containing 0.5 × 10^{-6} M rhodamine-labeled phalloidin (*Sigma*, St. Louis, USA) dissolved in methanol (Jung *et al.* 1991). Nuclei were visualized with 4,6-diamidino-2-phenylindole - DAPI (10 mg dm^{-3}). Stained cells were embedded in low melting agarose (*Sea Plaque, FMC Corporation*, Rockland, USA) and mounted on slides.

Microtubules in fixed suspension cells were stained with anti α-tubulin monoclonal antibody as described in Binarová *et al.* (1993). Cultured cells were fixed with 3.7 % paraformaldehyde in microtubule stabilizing buffer (MTSB: 50 mM piperazin-N,N`-bis(2-ethanesulfonic acid), 1 mM $MgSO_4$, 2 mM ethylene glycol-bis (β-aminoethylether)-N,N,N`,N`-tetraacetic acid, pH = 6.9) supplemented with 0.1 % Triton X-100. Samples washed in MTSB buffer were treated with enzyme (0.5 % cellulase, 0.25 % pectinase, 0.25 % driselase, 0.25 % macerozyme, 0.5 M manitol, 5 mM $CaCl_2$, 0.3 mM phenylmethylsulfonyl fluoride in MTSB buffer) and mounted on poly-L-lysine-coated slides. After washing in PBS (buffer saline) and blocking by 1 % bovine serum albumin in PBS buffer, slides were incubated with anti-α-tubulin monoclonal antibody DMA1 (*Sigma*). After washing in PBS buffer, secondary antibody goat anti-mouse-FITC conjugated (*Sigma*) was applied for 45 min. Nuclei were stained as above.

Microscopic observation was carried out on *Olympus* universal microscope equipped with epifluorescence optics (model *BX 60, Olympus Optical*, Hamburg, Germany). Data were recorded on *Kodak - T MAX 400* film.

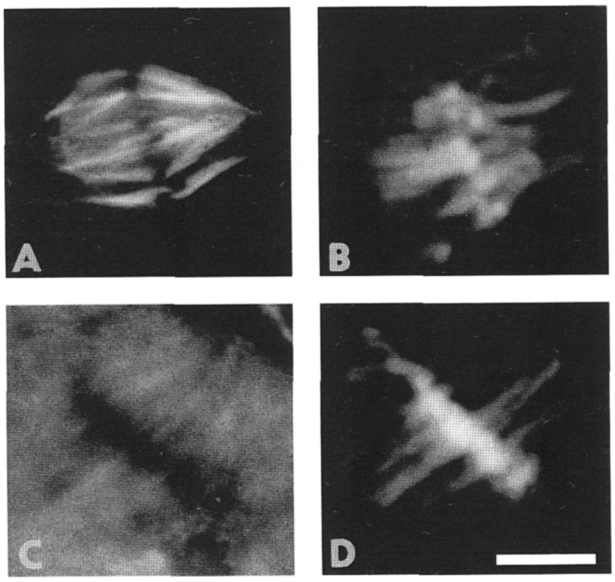

Fig. 2. Somatic embryogenic cells of spruce - metaphase: Actin staining with rhodamine phalloidine (*C*), tubulin staining with FITC conjugated antibody (*A*), DAPI staining of chromatin (*B,D*). Microtubular spindle consist almost exclusively formed kinetochore bundles of microtubules, which are focused to poles (A,B); microfilaments were absent in association with metaphase spindle (*C,D*). *Bar* = 10 μm.

Results

A very fine dense meshwork of randomly oriented actin microfilaments was observed in small rapidly dividing embryonal cells characteristic with the presence of big nuclei and dense cytoplasm (Fig. 1*A,B*) and with extensive network of randomly oriented cortical and cytoplasmic microtubules (Fig. 1*C,D*). Dense interphase

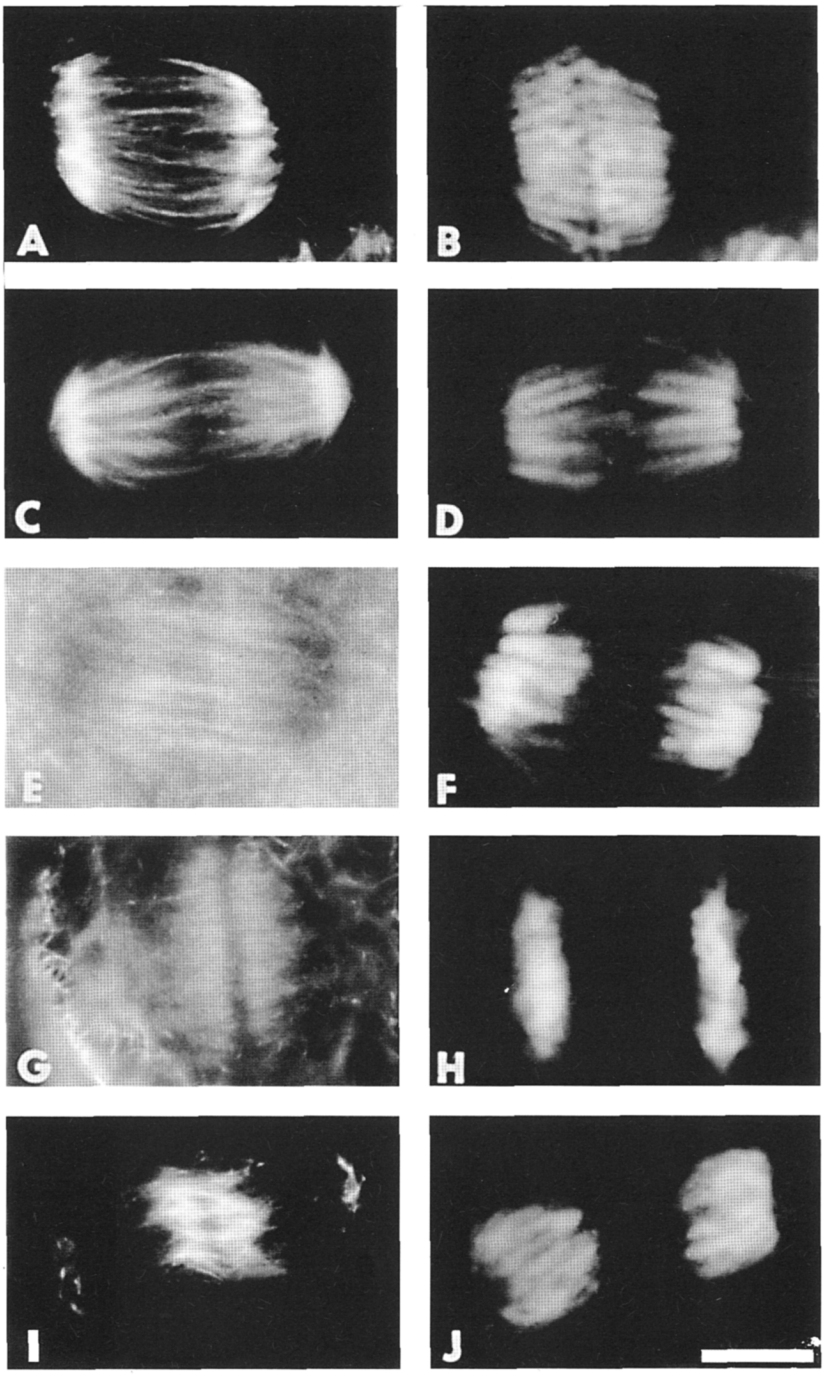

Fig. 3. Somatic embryogenic cells of spruce - anaphase. Actin staining with rhodamine phalloidine (*F,G*), tubulin staining with FITC conjugated antibody (*A,C,I*), DAPI staining of chromatin (*B,D,F,H,J*). During early anaphase the kinetochore microtubules are shortened considerably and numerous continuous microtubules were observed from pole to pole (*A,B*), anaphase microtubular spindle between separating chromatids is fully developed and consists of short kinetochore microtubules at the poles and long continuous microtubules between separating chromosomes (*C,D*), microfilaments were not observed to be localized along short kinetochore microtubules on poles of anaphase cells and fine anaphase microfilaments were localized only between separating chromosomes (*E,F*), later in anaphase long microfilaments shortened and formed very dense wide spindle-like structure associated with typical phragmoplast-like aperture in equatorial plane, which is similar to microtubular arrangement during phragmoplast formation (*G,H*), during anaphase soon after chromatids separation microtubules occurred also between separating chromosomes and they formed dense structure later establishing phragmoplast (*I,J*). *Bar* = 10 μm.

microfilament arrays disappeared when cells progress to mitosis. Cortical and subcortical microfilaments were found in prophase as a band located in equatorial level of the cell in the place where preprophase band (PPB) of microtubules is known to be localized (Fig. 1*E,F,G*). Actin PPBs occurred only in low percentage of cells, while microtubular PPB was regular part of the preprophase cells (Fig. 1*H,I,J*). As compared to cortical band of microtubules forming PPB, microfilaments were observed not only on the cell cortex but also deeper in the cytoplasm. Cells with well developed microtubule PPB were also characterized by intensive perinuclear fluorescence of tubulin and fine network of microtubules around nuclear envelope was focused to pole, forming prophase spindle (Fig. 1*H,I,J*). Co-localization of actin microfilaments with prophase spindle was not observed.

Cortical microtubules were absent during whole mitosis. During metaphase the chromosomes were aligned on the metaphase plate and the microtubular spindle consists almost exclusively from kinetochore microtubule fibers, which were focused to the poles (Fig. 2*A,B*). Microfilaments were absent or present only in a small amount in association this spindle structure (Fig. 2*C,D*). As compare to relatively stable cortical microfilaments observed in interphase cells, fine network of mitotic cortical microfilaments was a very sensitive to damage during staining procedure. Only a very fine short network of microfilaments, and thicker microfilaments or dots of actin were observed on cortex of metaphase cells (Fig. 2*C,D*).

At an early anaphase the kinetochore microtubules shortened and numerous continuous microtubules were located from pole to pole (Fig. 3*A,B*); later fully developed anaphase microtubular spindle between separating chromosomes occurred (Fig. 3*C,D*). Microfilaments were not localized with short kinetochore fibers at the poles of anaphase cells; fine dense anaphase microfilaments were localized only between separating chromosomes (Fig. 3*E,F*). In later anaphase midzone microfilaments shortened and formed very dense wide spindle-like structure associated with typical phragmoplast-like aperture in equatorial plane (Fig. 3*G,H*). While dense actin microfilaments were observed on the cell cortex in anaphase (Fig. 3*G,H*), cortical microtubules were still absent and only remnants of kinetochore microtubule fibers were present on the poles (Fig. 3*I,J*).

In early telophase actin spindle was completely rearranged into the actin phragmoplast structure. As compared to dense microtubular phragmoplast, which grew centrifugally to the cell wall (Fig. 4*A,B*), microfilaments were observed very often more in the center of the cell plate area (Fig. 4*C,D*). During later telophase actin microfilaments were observed in phragmoplast area only sporadically and actin was mainly visualized as diffuse area associated with microtubular phragmoplast on site of cell plate formation (Fig. 4*C,D*).

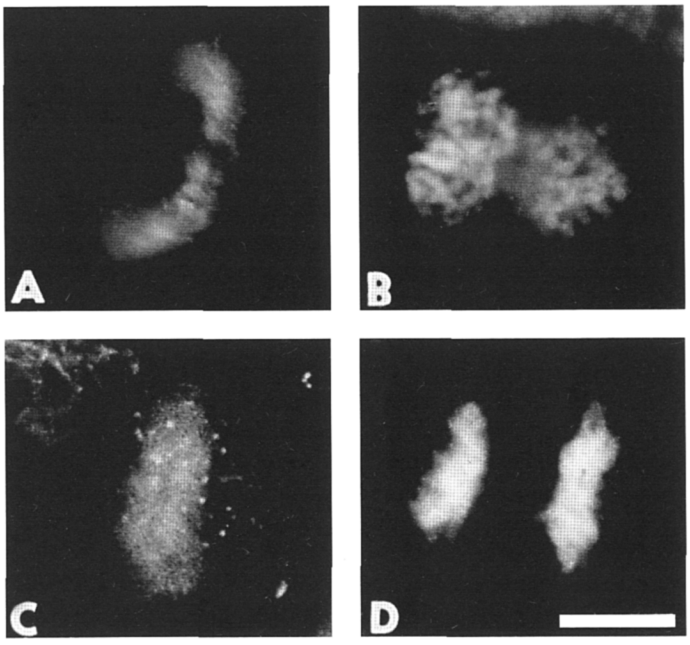

Fig. 4. Somatic embryogenic cells of spruce - telophase: Actin staining with rhodamine phalloidine (*C*); tubulin staining with FITC conjugated antibody (*A*); DAPI staining of chromatin (*B,D*); microtubular phragmoplast, which growth centrifugally to the cell wall (*A,B*); during later telophase actin was observed only as a diffuse staining associated with phragmoplast with narrow aperture in equatorial plane, the actin phragmoplast structure was observed only in the center of cell plate area (*C,D*). *Bar* = 10 μm.

Discussion

Microtubular cytoskeleton arrays during cell division of some conifer somatic embryos were described for different species: *Larix* sp., *Picea abies, P. glauca, P. mariana, Pinus* sp. (Fowke *et al.* 1990, 1995, Wang *et al.* 1991, Tautorus *et al.* 1992, Staxén *et al.* 1994). In agreement with their observation similar microtubular structures were observed in *P. abies* early somatic embryo cells in our experiments. Actin cytoskeleton of conifers was also characterized well (Hakmann *et al.* 1987, Fowke *et al.* 1995, Binarová *et al.* 1996). Only a few scattered microfilaments were observed in cells of embryo head, in contrast with suspensor cells, which contains numerous thick bundles of longitudinally oriented microfilaments. Fine actin network can be detected usually in some peripheral cells of embryonal group and these microfilaments are evident in extreme periphery of the cytoplasm in *P. glauca* and *P. mariana* (Hakmann *et al.* 1987, Fowke *et al.* 1995). Similar arrangement of interphase microfilaments was observed in suspension culture of Norway spruce early somatic embryos in our experiments. Co-localization of microtubules and microfilaments is well described in the pollen tube of some plant species (Terasaka *et al.* 1994). On the other hand, in meristematic cells of root of *Allium cepa* and *Tradescantia virginiana* cortical microfilaments have usually occupied the entire surface and were aligned parallel to the cortical microtubules (Liu *et al.* 1992). Despite the fact that double localization for actin and tubulin was not done in our experiments, from our numerous observation of microfilaments and microtubules on parallel slides, we can concluded that there was no clear co-localization of cortical microtubules and microfilaments in tubular suspensor cells or in isodiametric embryonal cells.

Actin microfilaments distribution or actin function in mitotic spindle of higher plants, are still not well understood. Controversial data about the presence of F-actin in mitotic apparatus of higher plants might be due to the differences in technique used for actin visualization. The majority of positive observations of actin microfilaments in spindle were obtained with unfixed cells stained by the fluorescent phallotoxins (Kakimoto *et al.* 1987, Jung *et al.* 1991, Czaban *et al.* 1993, Goddard *et al.* 1994, Binarová *et al.* 1996); the same technique was used in our experiments.

Preprophase band (PPB) - a specific cytoskeletal array in plants, is ring of cortical microtubules that marks the division site at which the cell plate forms during cytokinesis (Goddard *et al.* 1994). PPB appears in late S-phase, narrows throughout G2-phase, and disappears during the nuclear envelope break down in prophase (Assaad *et al.* 1997). Actin microfilaments associated in parallel with microtubular PPB, were first described in BY2 cells of tobacco suspension, in carrot suspension culture cells and in epidermal and root tip cells of *Allium* sp. (Kakimoto *et al.* 1987). In our experiments, microfilaments in equatorial plane of preprophase cells were observed only for transient period and they were arranged parallel to the microtubules of PPBs. Similar observation was reported in cells of wheat, carrot and *Tradescantia virginiana* (McCurdy *et al.* 1990). Cleary (1996) reported that cortical microfilaments were associated with microtubules in PPB and after breakdown of nuclear envelope persist on the cell cortex. Similar persistence of microfilaments in PPB after nuclear envelope breakdown was not observed in our experiments. On the other hand, F-actin labeling associated with PPB was not observed in other species (Clayton *et al.* 1985, Staigler *et al.* 1987). Actin localization in PPB has not been also reported in any conifer species studied until now (Fowke *et al.* 1995, Binarová *et al.* 1996). However how actin microfilaments are involved in function of PPBs remains to be elucidated. The presence of cyclin dependent kinase in PPBs suggests cell cycle specific regulation of this specific cytoskeleton structure (Mineyuki *et al.* 1990, Colosanti *et al.* 1993).

Presence of F-actin in spindle in mitosis suggests an involvement in this process. On the other hand, only little or no actin was detected in mitotic spindle of root meristem cells of *Allium cepa, Tradescantia virginiana* and *Haemanthus* sp. (Kakimoto *et al.* 1987, Palevitz 1987). Schmidt *et al.* (1988) showed presence of cortical F-actin network and an elastic cage around the spindle in isolated plant endosperm cells of *Haemanthus* sp. Localization of actin spindle fibers between chromosomes and on the spindle poles suggests that microfilaments and microtubules interaction can produce forces that move chromosomes during mitosis (Seagull *et al.* 1987). Actin microfilaments were observed in mitotic spindles during pollen development of *Gasteria verrucosa* (Van Lammeren *et al.* 1989) and in the mitotic spindle of suspension tobacco cells (Kengen *et al.* 1995). Microfilaments may not perform a universal function in plant cell division or alternatively the absence of microfilaments may be an effect of fixation in dividing meristematic cells (McCurdy *et al.* 1990). Possibility of cytoplasmic contamination of open plant cells spindles by cytoplasmic actin is discussed by Butt *et al.* (1988). Only very fine actin signal associated with metaphase and anaphase kinetochore fibers was observed in our experiments. Unspecific association of cytoplasmic soluble actin pool with metaphase spindle cannot be excluded. On the other hand, very prominent actin labeling occurred in equatorial plane of anaphase cell, where microfilaments formed actin spindle between separating chromosomes. Later actin microfilaments shortened and they were transformed to a phragmoplast-associated structure. Similarly to our observation, Schmidt *et al.* (1988) described formation of a new population of actin microfilaments at the equatorial plane in anaphase spindle before phragmoplast formation. Abundance of

actin in late anaphase and later in telophase indicate its importantance during cytokinesis in plants.

Phragmoplast, which is formed by microtubules and membranous organelles in equatorial zone of dividing cells in late anaphase, plays central role in cytokinesis. Diffuse staining of actin in phragmoplast area was already shown in *Picea glauca* by Binarová *et al.* (1996). Possible roles for actin in phragmoplasts might be the transport of cell plate precursor vesicles toward the midplane, organization and expansion of phragmoplast. Microfilaments concurently co-distributed with microtubules in cell plate area are important in cytokinesis (Shibaoka *et al.* 1994). Microinjection of antibodies against profilin, an actin associated protein, prevented formation of microfilaments in phragmoplast in dividing stamen hair cells of *Tradescantia* sp. F-actin filaments co-distributed with microtubules in all stages of cytokinesis were observed in meristematic cells of *Allium* and in stomatal complex cells of winter rye (Staigler *et al.* 1994) as well as in carrot suspension culture and meristem cells of maize (Traas *et al.* 1987). Studies of drug effect on cytoskeleton suggested close association of F-actin and microtubules in phragmoplast (Palevitz 1987). F-actin in phragmoplast appears amorphous and does not resemble the distinct actin fibers during interphase.

We found that actin is present in mitotic apparatus of *P. abies* throughout whole mitosis. While in prophase and metaphase only weak labeling was associated with spindle microtubules. F-actin became much more abundant in anaphase spindle. The prominent presence of F-actin in late anaphase spindle and in phragmoplasts suggests its importance in plant mitosis and mainly cytokinesis.

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