INVESTIGATIONS INTO POSTTRANSLATIONAL MODIFICATIONS OF α-TUBULIN AND COMPONENTS OF MTOCS IN PLANT CELLS

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Graduate Studies and Research
in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Biology
University of Saskatchewan
Saskatoon

By
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Spring 1999

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of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by
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ABSTRACT

Microtubules are important components of the plant cytoskeleton. They are dynamic polymers of tubulin which play crucial roles in cell division, cell growth and differentiation and ultimately are responsible for the overall morphology of the plant. For each of these roles, specific microtubule arrays must be formed in a precise place and in coordination with other cellular events. In order to form these arrays, microtubules must be assembled in the correct position and then selectively stabilized. In spite of the importance of these microtubule arrays, little is known about the processes that assemble or stabilize microtubules in higher plant cells. Animals and fungi have distinct cellular organelles – centrosomes or spindle pole bodies – which nucleate microtubules; however, in plant cells this nucleating function seems to be associated with membranes. Animals also have well described posttranslational and post-polymerizational modifications of tubulin incorporated into stabilized microtubules; however, these modifications have been reported only sporadically in plant cells. In this study, antibodies raised against components found in animal centrosomes and antibodies raised against modified α-tubulin were used to probe plant cells in order to identify microtubule nucleating sites and to determine if posttranslationally modified tubulin is present in plant cells. One posttranslational modification, acetylation, was found in abundance in some conifer but not in angiosperm cells. Another modification, detyrosination, was not present in either angiosperm or gymnosperm cells. These are the first reports of studies of posttranslational modifications of tubulin in gymnosperm cells. The results reported in this study on centrosomal components indicated that their distribution in gymnosperm and bryophyte cells is similar to those reported in angiosperm cells; but that this distribution does not unambiguously identify particular plant microtubule organizing centres.

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LIST OF ABBREVIATIONS

Ac mts: acetylated microtubules
BSA: bovine serum albumin
CLSM: confocal laser scanning microscopy or micrograph
CHO: Chinese hamster ovary (cells)
DAPI: 4'-6-Diamidino-2-phenylindole · 2HCl
Δ2-tubulin: non-tyrosinatable α-tubulin
ddH_2O: double distilled water
DTAF: dichlorotriazinylamino fluorescein
EM: electron microscopy or micrograph
ER: endoplasmic reticulum
FRAP: fluorescence recovery after photobleaching
glu mts: detyrosinated microtubules
IF: intermediate filament
mAb: monoclonal antibodies
MAP: microtubule associated protein
MAPK: mitogen-activated kinase
MEK: mitogen-activated kinase kinase
MTOC: microtubule organizing centre
MtSB: microtubule stabilizing buffer
NE nuclear envelope
NTT: non-tyrosinatable tubulin
PBS: phosphate-buffered saline
PBSB: phosphate-buffered saline with bovine serum albumin
PBSBT: phosphate-buffered saline with bovine serum albumin with Tween
PMSF: phenylmethylsulfonyl fluoride
PIPES: 1,4-Piperazinediethanesulfonic acid
SPB: spindle pole body (yeasts)
2D blot: Western blot of a two dimensional PAGE gel
TEM: transmission electron microscopy or micrograph
tyr mts: tyrosinated microtubules

Chapter 1. General introduction to microtubule structure and dynamics

Microtubules have been described as the morphogenic tools in eukaryotic cells (Gunning and Hardham 1982). In higher plant cells, microtubules exist in four major cytoskeletal arrays: the interphase cortical microtubules, the preprophase band, the mitotic spindle, and the phragmoplast. The correct placement and function of microtubules in each of these arrays is crucial. The interphase cortical array, by orienting cellulose microfibrils in cell walls (Marchant 1982; Cyr 1994), plays a vital role in establishing and maintaining cell shape in plant cells (Hush and Overall 1991). The preprophase band is a girdle of microtubules that is formed before mitosis at the precise site that the cell plate will fuse with the parental cell wall (Gunning 1982). The proper formation and functioning of the mitotic spindle and the phragmoplast is necessary to ensure that normal, mononucleate daughter cells are the result of mitosis. Obviously, the regulation of these microtubule distributions is important both at the level of the cells, and for the overall form and function of the plant itself.

The regulation of the formation of microtubule structures in any organism takes place at a number of levels (Marc 1997). (1) The microtubules themselves have characteristics, such as an inherent polarity and instability, that are crucial to the formation of microtubule arrays (Marc 1997). (2) Microtubule associations with other proteins, including microtubule associated proteins (MAPs), are believed to modulate the stability of microtubules by cross-linking them to each other or to other organelles. A subgroup of MAPs called microtubule motors can not only move organelles along microtubule "tracks", but also move the microtubules themselves. Therefore, the selective stabilization of certain microtubules by MAPs and/or the translocation of polymerized microtubules by microtubule motors could be important steps in the formation of a microtubule array (Vaughn and Harper 1998). (3) Microtubule organizing centres (MTOCs) control the site of microtubule assembly or nucleation, influence the number of microtubules formed at a particular time, and ensure that all microtubules formed at a particular site have the same polarity (Vaughn and Harper 1998).

The arrays of microtubules are integrated both spatially and temporally with other cell processes. An example of a spatial relationship is the close association of the microtubule portion of the cytoskeleton with actin microfilaments, another cytoskeletal component (Traas et al. 1987; Seagull 1989). Microtubules also have a close association with the plasma membrane and the cell wall because the cortical microtubules are believed to direct the orientation of cellulose microfibrils (except perhaps in tip-growing cells) that in turn controls the direction of cell expansion and the overall morphology of the plant (reviewed in Giddings and Staehelin 1991; Cyr 1994). In fact, the plasma membrane and the cell wall are believed by some to form a unit with the microtubules acting as strain gauges (Cyr 1994). This unit could be responsible for signal transduction and may be one way in which plant hormone signals (indirectly) and biophysical signals (more directly) change the orientation of cortical microtubules and also influence plant morphology (Cyr 1994). This theory also incorporates recent research on the plant primary cell wall that indicates it is a much more dynamic, responsive matrix than has been previously thought (reviewed in Carpita and Gibeaut 1993). Evidence of a functional relationship between the cortical microtubules and the cell wall includes findings that poly-L-lysine applied to the outside of protoplasts (plant cells with the cell wall digested away) stabilizes cortical microtubules (Akashi and Shibaoka 1991; Cyr 1994). The many cross bridges between microtubules and the plasma membrane (Gunning and Hardham 1982) could be specialized MAPs, providing one way that microtubules can be attached to membranes, although there are other possibilities for this connexion. Microtubules might associate with membranes through membrane tubulin (Gunning and Hardham 1982; Ludueña 1998) or by (as yet unidentified) integral membrane proteins (Akashi and Shibaoka 1991; Cyr 1994). The control of the formation of microtubule arrays is integrated temporally with the cell processes. The different arrays are coordinated with events in the cell cycle. How this is achieved is not well understood, although some information is accumulating. For example, the activities of MTOCs are influenced by the stage of the cell cycle. More microtubules are nucleated at centrosomes in mitotic compared to interphase cells (Marc 1997).

1.1 Microtubule structure

Microtubules are not permanent structures. In fact, their capacity to assemble and disassemble in response to subtle cellular conditions is crucial to their function. Microtubules are composed of rather stiff, hollow tubes of tubulin, with associated proteins (Gelfand and Bershadsky 1991; Fosket and Morejohn 1992). The tubulin is organized into (usually) 13 protofilaments of dimers, each dimer consisting of one α and one β subunit. The α and β subunits are flexible globular proteins with a molecular mass near 50kDa each and are held together by hydrophobic interactions (Fosket and Morejohn 1992). In fact, all of the bonds in microtubules are hydrophobic; the strongest are those holding the monomers together, the weakest holding the protofilaments together (Fosket and Morejohn 1992). Because the disassociation constant for the dimer is very low, tubulin that is not in microtubules exists in this form (Fosket and Morejohn 1992).

1.1.1 Tubulin heterogeneity

Multi-gene families code for both α- and β-tubulin; α- and β-tubulins in divergent species share 62% and 63% amino acid identity (Oakley 1995). Isotypes of these tubulins have highly conserved regions even between kingdoms (Fosket and Morejohn 1992). These conserved regions form the binding sites for GTP (Ludueña et al. 1992) and for α to β subunits (Fosket and Morejohn 1992) explaining why tubulin from very different sources can assemble into functional microtubules (Zhang et al. 1990).When looking at the amino acid sequences in either α- or β-tubulin, there can be as much variation within a species as between species (Fosket and Morejohn 1992). The C-terminus of the α subunit is the most variable region in the tubulin dimer (Fosket and Morejohn 1992). The variable C- terminus modulates the polymerization of tubulin *in vitro*, and participates in the binding of many MAPs (Rüdiger and Weber 1993; Ludueña 1998) that confer biochemical variability to the relatively uniform microtubules (Gelfand and Bershadsky 1991). A third member of the tubulin superfamily, γ-tubulin, is approximately 35% homologous to both α- and β-tubulins (Burns 1995) and is a key functional ubiquitous component of MTOCs, where it has a role in nucleating microtubules (Joshi and Palevitz 1996). It will be discussed in more detail in Chapter 4.

Alpha- and β-tubulin are similar to each other; they share 35-42% of their amino acid sequences with each other (Fosket and Morejohn 1992; Oakley 1995). In each species there are different isotypes of α- and β-tubulin, differing either genetically (coded on different genes) or by posttranslational modifications (Seagull 1989; MacRae 1997; Ludueña 1998). Genetic isotypes of angiosperm tubulins have 89% identity in amino acid sequences among α-tubulins and 87% among β-tubulins (Fosket and Morejohn 1992). Every plant examined to date has multiple tubulin genetic isotypes (e.g. Kopczak et al. 1992; Snustad et al. 1992 and reviewed in Ludueña 1998). These isotypes appear to differ in their tissue distribution (Carpenter et al. 1992; Ludueña 1998) and some are expressed preferentially in response to certain environmental conditions such as cold temperature, anoxia and continuous light (reviewed in Ludueña 1998).There has been a long-standing debate about whether the different genetic isotypes encode functionally different tubulins; this expression of different tubulin genes, presumably in response to certain environmental conditions would argue for some adaptive differences (discussed in Ludueña 1998). Furthermore, certain isotypic differences have been preserved over long periods of evolution resulting in isotype classes that have very similar sequences among all vertebrates even though there are considerable differences between class sequences in one cell (Ludueña 1998). There are conserved classes present in both monocots and dicots. This conservation of specific isotype class sequences suggests that different isotypes have different functions (Ludueña 1998). In support of this, certain tissue-specific genetic isotypes seem to be absolutely required for certain functions (Wilson and Borisy 1997). For example, if a *Drosophila* testis-specific β2-tubulin isotype is replaced by the somatically expressed β3-tubulin isotype, meiosis does not occur and the flagella axoneme does not form (reviewed in Wilson and Borisy 1997; Ludueña 1998). However, in many organisms and cells, genetic isotypes are functionally interchangeable and all can co-assemble into apparently normal interphase and mitotic arrays (Ludueña 1998) although the formation of a functioning axoneme seems to have more rigid requirements that are just beginning to be understood (Wilson and Borisy 1997; Ludueña 1998).

Different tubulin isotypes are also the result of posttranslational modifications (Sullivan 1988; MacRae 1997). Two of these modifications, acetylation and detyrosination, will be described in Chapters 2 and 3 in some detail. They involve reversible modifications to α-tubulin only and are both found in stable microtubules in a variety of organisms. Other posttranslational modifications include the covalent attachment of chains of either glutamate residues (polyglutamylation) or glycine residues (polyglycylation) to a glutamate near the C-terminus of either α-or β- tubulin (MacRae 1997; Ludueña 1998). The enzymes responsible for all of these posttranslational modifications have polymerized microtubules as a preferred substrate so they are post-polymerizational modifications as well (MacRae 1997). This is not the case with the phosphorylation of tubulin in which both dimeric and polymerized tubulin and both α - and β- tubulins are modified by a number of different kinases (reviewed in MacRae 1997). The function of these modifications is not clear, although along with genetic isotypes and the interaction with MAPs, they do contribute to the heterogeneity of the microtubule wall. Interestingly, certain genetic isotypes seem to be preferentially posttranslationally modified and some modifications seem to influence binding by MAPs, both structural and microtubule motors (Ludueña 1998). One of the objectives of this study was to look for the presence and distribution of two of these posttranslational modifications of α-tubulin, acetylation and detyrosination, in plant cells, in order to determine if these contribute to tubulin diversity in plant cells.

1.2 Microtubule Dynamics

Microtubules are dynamic structures, sensitive to minor changes in the state of their surroundings that drive assembly or disassembly (Lloyd and Barlow 1982). During *in vitro* polymerization, when the concentration of tubulin is above a critical concentration, microtubules can self assemble and exhibit treadmilling: adding dimers to a fast growing end (the plus end), and losing them from the other (the minus end) (Gunning and Hardham 1982). One molecule of GTP is hydrolyzed to GDP for each dimer incorporated into the microtubule, and this GDP cannot be exchanged for GTP while the tubulin exists in the microtubule (Hotani and Miyamoto 1990). Again during *in vitro* polymerization experiments, when a nucleating structure is provided, the microtubule's minus end is anchored in the nucleating material, and the microtubules display a kind of steady state called dynamic instability where slowly growing microtubules co-exist with rapidly shrinking ones (Morejohn 1991; Fosket and Morejohn 1992). It is believed that the addition of tubulin dimers to the plus end is stabilized by a GTP cap because the hydrolysis of GTP is a few steps behind the addition of the dimers. If the GTP cap is not maintained (i.e. the GTP is hydrolyzed faster than new dimers are added) the microtubule catastrophically depolymerizes, although rescue and regrowth of rapidly depolymerizing microtubules is possible (Gelfand and Bershadsky 1991; Hotani and Miyamoto 1990). Recent findings indicate that a protofilament capped with GTP is straight but one capped with GDP is curved and it is the curving away of GDP capped protofilaments that causes the microtubule to depolymerize (reviewed in Downing and Nogales 1998).

Although microtubules, found in all eukaryotic cells, share the same basic structures, different populations of them, even in the same cell, sometimes respond differently to cold (Marchant 1982), pressure (Cleary and Hardham 1990), ion concentrations (Wolniak 1988), and various drugs and herbicides (Morejohn 1991). It is the creation and regulation of this differential stability of microtubules that is the basis of the regulation of microtubule dynamics.

In animal cells, labile interphase microtubules turnover with a half time ($t_{1/2}$) of 5-

20 minutes while stable microtubules turnover with a $t_{1/2}$ of 1 or more hours (Black et al. 1989). This corresponds to the relative sensitivities to depolymerizing drugs (Black et al. 1989). Neurons have only stable microtubules with $t_{1/2}$ greater than 1.5 hours, half of these more than 5 hours (Black et al. 1989). They take up to 7-10 hours to depolymerize with depolymerizing drugs (Black et al. 1989). Even though they exhibit more cross-bridging than animal microtubules (Hardham and Gunning 1978; Seagull 1989), plant cell cortical microtubules turn over more quickly than animal interphase cells with $t_{1/2}$ of about 1 minute (Hepler and Hush 1996). Mitotic microtubules in both plant and animal cells turnover even more quickly with a $t_{1/2}$ of about 30 seconds for plant cells (Hepler and Hush 1996).

1.2.1 Modulation of microtubule dynamics

There is good evidence that MAPs affect microtubule dynamics. Animal tubulin with MAPs tend to treadmill, while those polymerized without MAPs tend to exhibit dynamic instability (Morejohn 1991). MAPs may be responsible for some of the properties of certain microtubule populations. For example, MAPs purified from carrot suspension cells, but not those purified from mammalian brains, conferred cold stability and resistence to destabilization with colchicine to neuronal microtubules (Cyr and Palevitz 1989). However, the effect of MAPs and their ability to confer stability is a complicated issue because in another set of experiments both cod and bovine MAPs seemed to be able to confer cold stability on microtubules even though cod, but not bovine, microtubules can assemble at cold temperatures. Atlantic cod, *Gadus morhua*, brain tubulin can assemble at temperatures as cold as 14°C during *in vitro* experiments but this assembly is dependent on MAPs at low temperatures (Wallin et al. 1993). Bovine MAPs had the same effect as cod MAPs, suggesting that the ability to polymerize at such cold temperatures must reside in the tubulin itself, not the MAPs (Wallin et al. 1993). MAPs may influence microtubule dynamics by stabilizing microtubules by attaching them to membranes or to each other

(Gunning and Hardham 1982; Vaughn and Harper 1998).

It has been suggested that the state of components of the MTOC, specifically the conformational state of γ-tubulin, may also influence the degree of dynamic instability of the microtubules assembled at this MTOC (Burns 1995).

1.3 Microtubule nucleation and organization

The concept of the MTOC includes: (1) a nucleating capacity, which involves a template function, determining the number of protofilaments and facilitating the polymerization of microtubules; (2) a capping of the minus end, that limits depolymerization at that end; and (3) orientation of the microtubules (Vaughn and Harper 1998). Whereas these functions are usually provided by the centrosome in animal cells, at least the nucleating function seems to be performed at membranes in plant cells. From observations of fluorescent analog experiments and microtubule recovery after depolymerization experiments, both the nuclear envelope and the plasma membrane are believed to nucleate microtubules in higher plants although the timing of this capability is probably regulated according to the stage in the cell cycle (Hepler and Hush 1996; Hasezawa et al. 1997; Vaughn and Harper 1998). Endomembrane material is also present at spindle poles where it may nucleate and /or cap microtubule minus ends (Vaughn and Harper 1998).

Recent studies have demonstrated that microtubule motors may play a central role in organizing mitotic spindles, both those with and without centrosomes (reviewed in Merdes and Cleveland 1997). The nucleating and organizational functions may therefore be separate processes with microtubules nucleated and even assembled by one process and then organized by motors (Marc 1997). Even in animal cells there can be separation between the nucleation and organization of microtubules. In epithelial cells, microtubules appear to be nucleated at the centrosome, then translocated and docked at a remote site (Mogensen et al. 1997; Tucker et al. 1998). This possible separation of nucleation and organization calls into question results identifying MTOCs by the location of converging microtubules in fixed cells without other corroborating evidence. Other lines of evidence indicating that a particular site is an MTOC could include (1) the presence of components found in other MTOCs, especially γ-tubulin, at the site; and, (2) the reassembly of microtubules at the site after complete depolymerization of microtubules in the cell (Marc 1997).

The second objective of this study was to identify MTOCs or nucleating sites in plant cells. In order to accomplish this, antibodies to three centrosomal components including γ-tubulin were used to probe plant cells. We also attempted to locate a phosphoepitope that is associated with MTOCs in animal and lower plant cells, in a conifer and a bryophyte by probing these cells with the antibody, MPM-2.

1.4 Differences between plant and animal microtubules

In the following chapters, each topic is introduced with a brief overview of the state of research in animal cells. Even though one of the central concepts in the study of the cytoskeleton, the MTOC, was first proposed in relation to plant cells (Pickett-Heaps 1969 cited in Vaughn and Harper 1998), more information is available about animal MTOCs, and posttranslational modifications of tubulin in animal cells. This is due to a number of factors that facilitate the study of the cytoskeleton in animal cells, such as the presence of an identifiable MTOC, the centrosome, and the availability of relatively large quantities of animal tubulin from mammalian brain cells. It is due as well to certain features of plant cells that make them relatively more difficult to study such as the presence of a rigid cell wall that is impenetrable to antibody probes, and fragile vacuoles filled with enzymes.

Even though it is useful to be aware of findings in animal cells when investigating the plant cytoskeleton, there are a number of differences between plant and animal microtubules that must be noted. These include differences between plant and animal tubulins themselves. Plant α-tubulins migrate faster on SDS gels than animal α-tubulins although β-tubulins from both co-migrate (Seagull 1989; Fosket and Morejohn 1992). Comparisons between plant and animal consensus sequences for plant and animal α-tubulins show four nonconservative substitutions in the exposed zone apart from the hypervariable C-terminal region. In spite of this, plant and animal tubulin can co-assemble to form functional microtubules (Fosket and Morejohn 1992).

Plant and animal microtubules react differently to some anti-microtubule depolymerizing compounds. Colchicine forms a complex with unpolymerized animal tubulin dimers, and this results in the depolymerization of animal microtubules (see discussion in Morejohn 1991). Plant microtubules are more resistant to the anti-microtubule effect of colchicine apparently because colchicine has a much lower affinity for plant tubulin than animal tubulin (Morejohn 1991). Plant microtubules are vulnerable to a range of pre-emergence herbicides have no effect on animal microtubules (Seagull 1989; Morejohn 1991).

In addition to these differences, plant microtubules form unique microtubule arrays mentioned above. Four major microtubule arrays appear sequentially in plant cells (Gunning and Hardham 1982; Baskin and Cande 1990). The interphase cortical array, in which microtubules are associated with the plasma membrane, orients the cellulose microfibrils of cell walls. The preprophase band is a girdle of microtubules that predicts the placement of the new cell wall and thus the site of division. The mitotic spindle is initially formed around the intact nuclear envelope at prophase. This structure, augmented by other microtubules, becomes barrel shaped at metaphase and lacks well defined poles. Eventually an anaphase spindle forms that in conifers has focussed poles and polar caps of microtubules (Baskin and Cande 1990; Wang et al. 1991). After mitosis, the phragmoplast, composed of microtubules from the interzonal portion of the anaphase spindle and of newly polymerized tubulin, appears in the region where the cell wall develops during cytokinesis.

Because of the differences between plant and animal tubulin, microtubules and microtubule arrays, it is important to study microtubules from both groups. Information on plant microtubules not only will contribute to our understanding of how the cytoskeleton functions in plant cells, but also will contribute to our knowledge of microtubules more generally.

1.5 Objectives

In order to understand if posttranslational modifications contribute to tubulin diversity in plant cells, the first objective of this study was to use immunofluorescence methods to look for the presence and distribution of two posttranslational modifications of α-tubulin, acetylation and detyrosination. Because both of these modifications chemically mark stable microtubules in animal cells, the results of this study could also contribute to our understanding of the relatively more stable portions of plant microtubule arrays.

The second objective of this study was to identify MTOCs or nucleating sites in plant cells. In order to accomplish this, antibodies to three centrosomal components including γ-tubulin were used to probe plant cells. We also attempted to locate a phosphoepitope associated with MTOCs in animal and lower plant cells in a conifer and a bryophyte by probing these cells with the antibody, MPM-2.

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Chapter 2. Acetylation – a posttranslational modification of α-tubulin – is found in some plant cells

2.1 Introduction

Acetylation is one of a number of posttranslational modifications that contribute to the heterogeneity of tubulin. Acetylation is the process in which an acetyl group is reversibly but covalently attached to the ε-amino group of lysine 40 of α-tubulin; it was initially discovered in *Chlamydomonas reinhardtii* when the flagellar form of α-tubulin, named α3, was found to be a posttranslationally modified form of α1, the cytoplasmic α-tubulin (L'Hernault and Rosenbaum 1985). The distribution of this α-tubulin isoform has been extensively studied because of the development of a well characterised monoclonal antibody that recognizes this modification (LeDizet and Piperno 1987, 1991). The antibody, 6-11B-1, is very specific and recognizes acetylation only at lysine 40 (LeDizet and Piperno 1991). It has been used to identify acetylated microtubules (microtubules that contain acetylated α-tubulin) in a large range of organisms. LeDizet and Piperno (1991) tabulated much of the information available before 1991; Table 2-1 presents a summary of papers published since.

The enzyme responsible for acetylation has been partially purified and characterized, but there is less information about the enzyme responsible for the reverse process, deacetylation, and even less about a protein that acts as an inhibitor of acetylation (MacRae 1997). Acetylation is performed on α-tubulin by an acetyltransferase that acts preferentially on polymerized tubulin (Maruta et al. 1986; Black et al. 1989). This acetylase acts at multiple sites along microtubules and binds to axoneme microtubules in a Mg^{++} dependant way (Maruta et al. 1986; MacRae 1997). Tubulin acetyltransferase has been purified from both *Chlamydomonas* and mammalian brain and it has been detected in

Table 2.1 Structures stained with 6-11B-1 antibody raised against acetylated α-tubulin

Organism	Structures stained	Reference
VERTEBRATE ANIMALS		
human	prostrate cancer cells: more Ac* mts* in a cell line resistant to Estramustine (an antimicrotubule drug)	Sangrajang et al. 1998
	H69 human small cell lung cancer line: more Ac mts in a taxol resistant cell line than in original cell line	Ohta et al. 1994
brain mts	hydrophobic mts associated with membranes highly acetylated	Nunez-Fernandez et al. 1997
mouse	testis: axoneme, centriole, +/- midbody	Fouquet et al 1994
	little Ac tubulin in testis in mutant mice with no germ cells	Wolf and Winking 1996
	sperm flagella uniformly labelled	Fouquet et al. 1996
	cultured fibroblasts: Ac mts associated with Glu mts, both grouped close to the MTOC and Golgi	Thyberg and Moskalewski 1993
rat	testis: axoneme, centriole, +/- midbody	Hermo et al. 1990
	rod photoreceptors: all mts of the connecting cilium at all stages contained Ac mts	Arikawa and Williams 1993
	L-6 muscle cell levels of acetylation increased during differentiation, on stable mts	Gundersen et al. 1989
	cultured neurons: majority of α-tubulin in mts is acetylated	Black and Keyser 1987, Black et al. 1989
	cerebellar macroneurons: acetylation restricted to axons in early growth, distributed in axons and dendritic trunks in mature neurons; acetylation correlated with colchicine resistance	Ferreira and Caceres 1989
turkey	erythrocyte marginal band: no acetylation	Rüdiger and Weber 1993
Gadus morhua	only in mts from central and peripheral nervous system, no other tissue	Rutberg et al. 1995
	subpopulation of mts in melanophores	Nilsson et al. 1996
Gymnocorymbus ternetzi	melanophores: in few mts	Rodionov et al. 1994
Xenopus laevis	egg and early embryo: meiotic and mitotic spindles, midbodies, but not the cortical mts or sperm asters contain Ac tubulin; after gastrulation, Ac mts are in only midbodies; later it is found in neuronal tissue	Chu and Klymkowsky 1989
	oocytes: a number of mts are acetylated	Gard 1991
INVERTEBRATE ANIMALS		
Heligmosomoide polygryus	spermatocytes and spermatids: no acetylation	Mansir and Justine 1998
Tenebrio molitor	meiosis: all mt arrays acetylated to some degree, proportion of mts acetylated increases throughout meiosis I	Wolf and Hellwage 1995
	mitosis: all mitotic mt arrays highly acetylated	Wolf and Hellwage 1995
Pyrrhocoris apterus	meiosisI: intensely stained in metaphase I and anaphase spindles (holokinetic); less intensely in late telophase I	Wolf 1996a
Oncopeltus fasciatus	ovarian nutritive tubes: weak staining	Wilson and Forer 1989
Notonecta glauca	ovarian nutritive tubes: staining of older tubes	Wilson and Forer 1989
Ephestia kuehniella	interzonal mts in telophase I; no other structure including basal bodies	Wolf 1996a
	all mitotic stuctures highly acetylated	Wolf 1994, 1996b

Pieris brassicae	meiosis: all mt arrays acetylated to some degree, proportion of Ac mts increases throughout meiosis I mitosis: all mitotic arrays highly acetylated	Wolf 1996b
Nephrotoma suturalis (Loew)	cells in all stages of spermatogenesis: acetylation faster in spindles than in non-dividing cells	Wilson and Forer 1989
	kinetochore fibres selectively stained in spindles	Wilson and Forer 1989; Wilson et al. 1994
Artemia	larvae: all mt structures including mitotic spindles and midbodies	MacRae et al. 1991
Platyhelminthes	Ac tubulin in axonemes only	Iomini et al. 1995
PROTISTA		
Giardia lamblia	no non-Ac α-tubulin detected by automated sequencing of CNBr fragments	Weber et al. 1997
Trichomonas vaginalis	all interphase arrays contain Ac mts; Ac mts also found in the extranuclear mitotic spindle (paradesmosis) and axostyles	Delgado-Viscogliosi et al. 1996
Tetrahymena thermophila	most or all ciliary tubulin is acetylated, but this is not necessary for mt function	Gaettig et al. 1995
Tryponosoma brucei brucei	Ac mts found in flagellum, subpellicular mts	Scnuto-Padron et al. 1993
Paramecium	Ac mts found in axonemes and other stable mts; little or no Ac mts in cytoplasmic mts	Bre et al. 1994
	Ac mts found in nocodozole resistant mts	Fleury et al. 1995
Plasmodium falciparum	no acetylation, although 6-11B-1 epitope can be produced *in vitro*	Read et al. 1993
FUNGI		
Allomyces arbuscula	more Ac tubulin in zoospores than in mycelium (immunoblots only)	Schonenberger and Turian 1993
Allomyces macrogynus	zoospores: axonemes, kinetosome and cytoplasmic mts	Aliaga and Pommervilee 1990
PLANTS		
algae		
Chlamydomonas reinhardtii	flagellar and cytoplasmic mts	LeDizet and Piperno 1986, Kozminski et al. 1993
Thalassiosira fluviatilis	no Ac tubulin	Machell et al. 1995
Pteridophytes		
Ceratopteris richardii	spermatogenous cells: herbicide resistant mts	Hoffman and Vaughn 1996
Ceratopteris richardii	spine, mts associated with the basal bodies and flagella	Hoffman and Vaughn 1996a, b
angiosperms		
Nicotiana tabacum	pollen tubes: Ac tubulin in the generative cell in interphase and mitosis	Aström 1992
	pollen tubes: no Ac tubulin	Del-Casino et al. 1993
	cultured cells: all mt arrays; spindles were labelled only at poles	Smertenko et al. 1997
Secale cereale L.	root tips: cortical phragmoplasts and preprophase bands but not spindles labelled	Kerr and Carter 1988

* Ac = acetylated; mt = microtubules

bovine retinal tissue (see MacRae 1997 for review). The enzyme acts preferentially on polymerized tubulin, making acetylation a postpolymerizational as well as a posttranslational modification (Maruta et al. 1986; MacRae 1997). This is demonstrated in a study of acetylated tubulin in neurons where 99% of the acetylated tubulin was in microtubules (Black et al. 1989). The presence of a tubulin deacetylase and an inhibitor of tubulin acetylation have also been demonstrated, but neither has been purified (Maruta et al. 1986). In the case of *Chlamydomonas reinhardtii*, tubulin acetyltransferase was found in the flagella, while the deacetylase and the tubulin acetylation inhibitor were located in the cytoplasm (Maruta et al. 1986). Deacetylation takes place either just before or just after depolymerization. Evidence for this is based on immunoblot results performed on samples from cultured mouse embryo cells where acetylated α-tubulin was detected in polymeric form only (Piperno et al. 1987), and on the finding that although treatment of neurons with depolymerizing drugs decreased the acetylation levels of polymer, there was no corresponding increase of acetylated soluble tubulin (Black et al. 1989).

The compilation by LeDizet and Piperno (1991) and Table 2.1 indicate that acetylation of α-tubulin is found in a range of organisms in all kingdoms. Its function (and that of other posttranslational modifications of tubulins) is still unclear. Acetylation is certainly not necessary for survival, as there are organisms with no acetylated tubulin, such as yeasts, which do not have a lysine 40 residue to acetylate (LeDizet and Piperno 1987). It is also absent in some organisms and cell lines, that do have a lysine 40 residue but no acetylation, notably *Plasmodium falciparum* (Read et al. 1993), PtK cell lines (Piperno et al. 1987), turkey erythrocytes (Rüdiger and Weber 1993) and the interphase cells of the diatom *Thalassiosira fluviatilis* (Machell et al. 1995). Mutants have been created in which more than half of the tubulin (*Chlamydomonas reinhardtii*, Kozminski et al. 1993) or all of the tubulin (*Tetrahymena thermophila*, Gaettig et al. 1995) is rendered nonacetylatable by replacing the lysine 40 of α-tubulin by another amino acid such as arginine. The fact that such mutants are indistinguishable from the wild type indicates that either the acetylation or the lysine is not essential; however acetylation of lysine may be essential if lysine is present (Kozminski et al. 1993; MacRae 1997). MacRae (1997) claimed that a critical test for the function of tubulin acetylation would be to prevent expression of the tubulin acetyltransferase. These experiments have not been done although the consequences of tubulin acetyltransferase impairment in a human male have been described (Gentleman et al. 1996). The patient had α-tubulin hypoacetylation (30% of control) and abnormally low tubulin acetyltransferase activity in his sperm (also 30% of normal). He also had a number of irregularities, all related to abnormal microtubule function: infertility as a result of impaired motility of the sperm, gross morphological abnormalities of the sperm axonemes, rod-dominant retinal degeneration, and neurological function impairment (Gentleman et al. 1996). This appears to be the only report of its kind, but if it is corroborated, it may indicate that tubulin acetylation is required for certain microtubule functions.

Acetylation may have some role in modulating microtubule function. There are three lines of evidence for this suggestion. (1) The primary sequence of α-tubulin surrounding the acetylation site at Lysine 40 is well conserved (Kozminski et al. 1993), and the site is probably exposed to the outside of the polymerized microtubule (Maruta et al. 1986). (2) Acetylation of tubulin occurs in what seems to be a regulated distribution. This regulation is sometimes spatial, as in the case of the compartmentalization of the acetylase and deacetylase enzymes in *Chlamydomonas* mentioned above, or it may be temporal, as in the case of the spermatogenous cells of the pteridophyte *Ceratopteris richardii* where acetylation occurs only late in spermatid development (Hoffman and Vaughn 1995a). (3) Acetylation correlates with microtubule stability in the presence of depolymerizing drugs in both mammalian cells and *Chlamydomonas* (LeDizet and Piperno 1986; Piperno et al. 1987; LeDizet and Piperno 1991). It also correlates with microtubules that are resistant to depolymerization by certain herbicides. In spermatogenous cells of the pteridophyte *Ceratopteris richardii*, microtubule arrays containing acetylated tubulin are stable and resistant to microtubule disrupting herbicides while all other arrays are sensitive (Hoffman and Vaughn 1996). The correlation of herbicide resistance and acetylation has also been noted in *Chlamydomonas* (James et al. 1993). Acetylated microtubules are not resistant to depolymerization by cold, which may be a less specific depolymerizing agent in that it affects almost all enzymatic reactions as well as water-protein interactions, protein-protein interactions and such basic processes as diffusion (LeDizet and Piperno 1986).

The modulation of microtubule function may occur in different ways. Acetylation could be one of a number of steps in the stabilization of microtubules (LeDizet and Piperno 1991), perhaps by affecting the binding of associated proteins (Laferrière et al. 1997). Acetylation may block a reactive site on polymerized α-tubulin (Kozminski et al. 1993) and "confer functional specificity to particular regions of stable microtubules and affect interactions with other cytoskeletal elements or organelles" (Webster and Borisy 1989). These interactions may involve specific associations with membranes. For example, in rat brain tissues, acetylated microtubules are preferentially associated with membranes (the plasma membrane as well as organelle membranes) (Beltramo et al. 1992).

2.1.1 Acetylation and stability

Whatever the role of acetylation, there is an association, whether causal or not, between acetylation and microtubule stability. Accumulating evidence indicates that acetylation of α-tubulin is not essential in creating or maintaining stable microtubules but may be instead a marker for microtubules stabilized in some other manner (MacRae 1992). In fact, acetylated tubulin in animal cells is often found in very stable microtubule structures such as axonemes of flagella, basal bodies, or microtubules in neurons (Ferreira and Caceres 1989; LeDizet and Piperno 1991; Fouquet et al. 1994; Iomini et al. 1995; Jackson et al. 1995; Rutberg et al. 1995; Fouquet et al. 1996; Mollaret and Justine 1997). Even in cells that do not have these structures, acetylated microtubules are more stable than other microtubules. In human fibroblasts, for example, acetylated microtubules have a markedly slower turnover rate than those microtubules that are not acetylated (Webster and Borisy 1989). This correlation has been observed in so many organisms that researchers often use 6-11B-1 as a marker for stable microtubules (eg Takemura et al. 1992; Smith 1994; Wolf and Spanel-Borowski 1995; Geyp et al. 1996; Hempen and Brion 1996; Rochlin et al. 1996; Graff et al. 1997; Pittman et al. 1997).

Acetylated tubulin is found in more labile microtubule structures of some cells such as the mitotic spindles of invertebrates (MacRae et al. 1991; Wolf and Hellwage 1995; Wolf 1996a, b) and some cultured vertebrate cells (Piperno et al. 1987; Draber et al. 1989). This is possible because acetylation can occur very quickly after polymerization, within five minutes in TC-7 cells (Bulinski et al. 1988), and 15 minutes in HeLa cells (Piperno et al. 1987). Even in these more labile microtubule structures, acetylation can be associated with relatively stable microtubules. This is illustrated in a series of experiments using crane fly meiotic spermatocytes (Wilson and Forer 1989; Wilson et al. 1994; Wilson and Forer 1997). Kinetochore microtubules in these cells are selectively stained with 6-11B-1, the antibody to acetylated α-tubulin, except for a gap next to the kinetochores (Wilson and Forer 1989). This led the researchers to suggest that tubulin subunits were added to the kinetochore fibres at the kinetochore and were subsequently acetylated as they aged and fluxed poleward (Wilson and Forer 1989). In a later set of experiments, Wilson and Forer (1994) were able to determine that the major site of microtubule disassembly was not the kinetochore, but the spindle pole. They did this by comparing the length of the gap in acetylation during metaphase I and anaphase I and determining that the length of this gap decreased during anaphase at a rate that was slower than chromosome movement (Wilson et al. 1994). In a further set of experiments, they ruled out the possibility that the persistence of the gap during anaphase I chromosome movement was due to an unusual wave of deacetylation occurring on the kinetochore fibres by treating the cells with taxol (which stabilizes microtubules), and observing that the gap in these treated cells did disappear as the microtubular dynamics of these cells was slowed (Wilson and Forer 1997). These cranefly experiments illustrate that certain relatively stable microtubules can be acetylated even in microtubule structures that are considered labile.

However, the association of stability with the degree of acetylation is sometimes not straightforward and instead the level of acetylation seems to be associated with enzyme kinetics, concentration of enzyme, or other factors. For example, in early embryos of *Xenopus* that undergo rapid cell division, mitotic spindles are acetylated. However, when division rates slow in later stages, only the midbody contains acetylated tubulin (Chu and Klymkowsky 1989). In some cells such as 3T3 and HeLa cells (Piperno et al. 1987), treating with taxol will result in the acetylation of all the microtubules, whereas in the spermatogenous tissue of *Ceratopteris richardii*, treating with taxol did not result in acetylation until late in spermatid development when acetylation normally occurs in certain structures (Hoffman and Vaughn 1996). This indicates that the acetyltransferase is not present in the fern cells at all times.

2.1.2 Acetylated microtubules in plant cells

There are only a few examples of acetylated tubulin in plant cells. When it is present in algae and ferns, acetylated tubulin seems to be restricted to stable microtubule structures. In *Chlamydomonas reinhardtii*, flagellar microtubules, basal bodies and a few drug resistant cytoplasmic microtubules contain acetylated tubulin (LeDizet and Piperno 1986). In fern spermatogenous cells, acetylated tubulin was found in the spline, basal bodies and flagella, all highly stabilized microtubule arrays (Hoffman and Vaughn 1995a, b).

There have been very few reports of the distribution of acetylated tubulin in higher plant cells, all in angiosperms. In one study of *Nicotiana tabacum* pollen tubes, acetylated tubulin was observed in the generative cell mitotic spindle and phragmoplast, as well as in microtubules associated with the generative cell nucleus (Åström 1992). When the pollen tubes were treated with taxol, very short fragments of microtubules also stained with the antibody to acetylated tubulin (Åström 1992). In another study using the same material, acetylated tubulin was not detected (Del-Casino et al. 1993). Acetylated microtubules were also recently reported in all microtubule arrays in *Nicotiana* cultured cells (Smertenko et al. 1997). Acetylated tubulin was also reported in preprophase bands and phragmoplasts but not spindles of rye root tip cells (Kerr and Carter 1988 abstract).

It is not known whether acetylated tubulin is present in gymnosperm or bryophyte cells, and the evidence about it in angiosperms is confusing. In the present study, the monoclonal antibody to acetylated tubulin (6-11B-1) was used in immunofluorescence staining of methacrylate semi-thin sections to study the distribution of acetylated tubulin in meristematic regions of four species of gymnosperms, and two species of angiosperms, and immunogold labelling of one bryophyte species. Individual whole cells of *Pinus radiata* and *Allium* root tips were also immunolabelled to determine the distribution of acetylated tubulin therein.

2.2 Materials and Methods

2.2.1 Plant material

Pinus radiata: Seeds (a gift from A. Hardham) were surface sterilized seeds in 30% hydrogen peroxide, rinsed in 5 changes of sterile ddH_2O, and then germinated on filter paper over vermiculite and grown at room temperature. Root tips (3-6 mm) of 9-12 day old seedlings were fixed as described below. *Pseudotsuga menziesii* (Mirb.) Franc.: surface sterilized seeds (from S. Misra, University of Victoria, sterilized as described above) were stratified by hydrating and storing for 21 days at 4°C, then germinated at room temperature. After a week, 5 mm root tips were excised and fixed. *Picea glauca:* whole somatic embryos matured for 3 or 8 weeks with abscisic acid and polyethylene glycol (Attree et al. 1995) were fixed. *Pinus contorta:* surface sterilized seeds (a gift from Saskatchewan Environment and Research Management Prince Albert Forest Nursery) were germinated on filter paper over vermiculite and grown for 10 days. One mm sections of root cap were discarded from the root tips and the next 4-5 mm were fixed. *Allium cepa* (cv. Walla Walla): surface sterilized seeds were germinated on filter paper over vermiculite. Root tips (3-6 mm) of 7-9 day old seedlings were fixed. *Helianthus annuus* (var. Peredovic): 14 day vegetative shoot tips were used. *Marchantia polymorpha*: the notch region of gemmlings (gemmae grown on soil under long days for 8 days) were excised into fixative.

2.2.2 Preparation of methacrylate sections

Methacrylate sections were prepared essentially as described by Baskin et al. (1992). Briefly, material was fixed in 4% methanol-free formaldehyde (Polysciences) and 0.2% glutaraldehyde in microtubule stabilizing buffer (MtSB: 50mM PIPES, 5mM EGTA, 2mM $MgSO_4$, pH 6.9), for 1.5 hr at room temperature, washed and dehydrated to 100% ethanol over 6 hours, in increments of 10%, 30%, 50%, 70% and 90% with 3 changes in 100% ethanol (10% to 50% ethanol on ice with higher concentrations of ethanol at -20°C). The samples were infiltrated with methyl/butyl methacrylate that was prepared as 1 part methyl methacrylate: 4 parts butyl methacrylate, 0.5% benzoin ethyl ether and 10mM dithiothreitol with oxygen displaced by bubbling nitrogen through it for 1 hour before use. Fresh methacrylate was diluted with ethanol in ratios of 1:2, 1:1 and 2:1 for sequential steps. Each step in the infiltration procedure was at least 4 hours, and this was followed by 2 changes of 100% methacrylate over 24 hours all at -20°C. Each root tip or embryo was transferred to an individual modified Beem embedding capsule (size 00, from J.B. EM Services Inc.) with a lid on each end, allowing the tissue to be aligned, and left in the dark at -20°C for at least 8 hours before exposure to UV light (10 cm from a 365 nm 20 W lamp) at -5°C. Polymerization was completed at +5° C. Polymerized blocks were stored in desiccant up to 2 years until sectioning. Sections, 1.5 - 2.5 μm in thickness, were collected from dry glass knives onto poly-L-lysine coated, welled slides (Polysciences) and air dried. Just before immunostaining, resin was removed in acetone (ultrapure) by shaking slides slowly for 10 minutes on a rotary shaker. The sections were moved to 100% ethanol and rehydrated in 4 steps to 30% ethanol in 0.85% NaCl (Kronenberger et al. 1993) before transfer to phosphate buffered saline (PBS) pH 7.3 for 5 minutes and blocking with PBSB (1% protease free BSA in PBS) for 20 minutes at room temperature.

2.2.3 Preparation of root tip cells

Pinus radiata, Allium cepa and *Pinus contorta* root tips, 3-6 mm long, were prepared as described by Wick (1993). Samples were fixed for 1-1.5 hours with 4% methanol-free formaldehyde (Polysciences) in MtSB containing 10% DMSO and 0.1% TritonX-100, followed by 3 washes of 10 minutes each in MtSB. Fixed root tips were digested for 1 hour (pine) or 30 minutes (onion) in 1% Cellulysin (Calbiochem) in either MtSB or 0.4M mannitol with 0.3mM PMSF and 19 mM leupeptin, then washed 3x20 minutes in MtSB. (No differences were observed between cells digested with enzymes in MtSB or mannitol). Root tips were squashed between two clean, welled slides (Polysciences) in a small drop of MtSB (Wick 1993), and the released cells were immobilized by drying onto the bare glass. The cells were extracted with 0.5% Trtion X-100 in MtSB for 20 minutes and washed 3x1 minute in PBS. To block unreacted aldehydes, cells were treated for 6 minutes with 0.1% sodium borohydride in PBS, and washed 2x5 minutes in PBS. Cells were preblocked with PBSB for 20 minutes at room temperature.

2.2.4 Immunofluorescence staining of methacrylate sections and isolated cells

For single staining, monoclonal antibodies (mAbs) against β–tubulin (Amersham) and acetylated tubulin (6-11B-1, Sigma) were diluted 1/250 and 1/200 respectively in PBSBT (PBSB with 0.5% Tween-20), applied to the sections or cells and incubated in a humid chamber at 4°C overnight and then at 37°C for 1 hour. Slides were washed with a stream of PBS, then in 3 changes of PBS over 1.5 hours. Negative controls were exposed to PBSB in place of the primary antibodies. Sections or cells were incubated in secondary antibody (goat anti-mouse Cy3 for conventional microscopy or Cy5 for confocal laser scanning- Jackson Immunoresearch) diluted 1/500 in PBSBT and incubated for 1 hour at 37°C in a moist chamber. Slides, washed as for the primary antibody, and then in double distilled water (ddH_2O) for 5 minutes, were counterstained with DAPI (2.5 μg/ml) in ddH_2O for 2 minutes, before washing in ddH_2O and mounting in Fluorsave (Calbiochem). Reference sections were stained with toluidine blue.

For double staining with tyrosinated tubulin and acetylated tubulin, an affinity purified polyclonal antibody to tyrosinated tubulin (Anti-Y, a kind gift from Dr. T.H. MacRae, Dalhousie University, Halifax) (diluted 1/100) was applied at least one hour after 6-11B-1. For samples to be viewed using a confocal microscope, secondary antibodies were Cy5 conjugated goat anti-mouse and DTAF (an FITC alternative) conjugated goat anti-rabbit (both from Jackson Immunoresearch). After immunolabelling, samples were briefly treated with RNase I (0.1mg/ml in PBS, incubated for 15 minutes 37°C), rinsed 2 times in PBS before staining with propidium iodide then washing and mounting as above.

For conventional fluorescent microscopy, slides were examined using a Zeiss Axioplan fluorescence microscope fitted with standard filters and a 63 x Apochromatic oil immersion objective lens. Ilford XP2-400 film was used for photography, and plates were assembled using Adobe Photoshop 5.0. For confocal microscopy, slides were examined using a Zeiss LSM 10 inverted laser scanning confocal microscope equipped with an argon- krypton laser with emission barrier filters of 488nm and 568nm. Whole cells were optically sectioned in a z-series of 1 μm sections separately for each of the wavelengths used. Results were consistent over at least 5 different experiments using at least 10 sections each time.

2.2.5 Preparation and immunogold staining of LR White sections

Marchantia gemmlings were fixed for 1 hour at room temperature in 1% methanol-free formaldehyde plus 1% glutaraldehyde in 0.1M PIPES buffer, pH 6.9, washed 3 times in the same buffer and then dehydrated through an ethanol series in 30 minute intervals as follows: 10% ethanol (room temperature), 30% (0°C), 50% (0°C), 70% (-20°C), 95% (-20°C) and 3 changes in desiccated 100% ethanol (-20°C). Tissue was infiltrated with LR White resin diluted with ethanol in ratios of 1:1 and 2:1. Each step in the infiltration procedure was at least 8 hours, and this was followed by 2 changes of 100% LR White of 1 day each; all steps of infiltration were at -20°C. Each gemmling was transferred to an individual modified Beem capsule with a lid on each end, allowing the tissue to be aligned, or a No. 1 gelatin capsule, overfilled with fresh resin to exclude oxygen, and left in the dark at -20°C for at least 8 hours before exposure to UV light (10 cm from a 365 nm 20 W lamp) at -10°C to polymerize. Polymerized blocks were stored in desiccant at room temperature until sectioning. Pale gold sections were collected onto formvar coated nickel grids, before immunolabelling.

During immunolabelling, the grids were floated on a series of solutions in droplets on welled slides in a humid chamber, at room temperature, and vibrated on an insulated aquarium pump. The grids were blocked on droplets of PBSB, 0.1% Tween, (10 minutes); PBSB with 0.02M glycine (3minutes); washed in PBSB 3 x 3 minutes; then incubated on primary antibody (1/100 dilutions in PBSB of monoclonal antibodies 6-11B-1 or B-5-1-2, an anti α-tubulin antibody – both from Sigma) for 1½ hours, then washed 3 x 3 minutes in PBSB before incubating on secondary antibody (1/20 dilution in PBSB of 10 nm gold-conjugated goat anti-mouse IgG from BioCell). Grids were washed 3 x 1 minute in PBS, then similarly in ddH_2O, and allowed to dry on filter paper.

Sections were counterstained 3 minutes with 1% uranyl acetate in 70% ethanol (with an ascending 10%, 30%, 50% alcohol series before and a descending series after), washed in a stream of filtered ddH_2O, and then in a petri dish of ddH_2O, followed by 3 minutes in Reynold's lead citrate stain. Specimens were photographed with a 420 model or CM-10 model Phillips electron microscope. Developed photographs were scanned on a Canon flatbed scanner and the figures were assembled using Adobe Photoshop 5.0.

2.3 Results

2.3.1 Distribution of acetylated tubulin in methacrylate embedded sections of root tips, somatic embryos, and shoot apices

Pinus radiata:

Root tips of *Pinus radiata* that were fixed, embedded in methacrylate and stained with toluidine blue exhibited a large number of dividing cells as shown in Fig. 2.1a. Results using the mAb against β-tubulin (Fig. 2.1b) were similar to those reported earlier, with staining of all four major microtubule arrays found in plant cells, namely cortical microtubules, preprophase bands, mitotic spindles and phragmoplasts (Fowke 1993). Acetylated tubulin was also detected in all microtubule containing structures in *Pinus radiata* sections, including phragmoplasts (Fig. 2.1c), prophase spindles (Figs. 2.1c, 2.1e), metaphase spindles (Fig. 2.1c) and anaphase spindles (Figs. 2.1d, 2.1e), as well as cortical microtubules (Fig. 2.1d). Recently divided cells often had a high level of stain in their cytoplasm (Fig. 2.1e). Negative controls lacking exposure to primary antibodies were unstained (not shown).

Other conifers:

Methacrylate sections of *Pseudotsuga menziesii* roots, whole *Picea glauca* embryos and *Pinus contorta* roots were well stained with the antibody to β-tubulin revealing the same structures seen in *Pinus radiata* (Figs. 2.2b, 2.2d, 2.2f). However, when compared to one another, microtubule arrays in these species exhibited different staining intensity with 6-11B-1. For example, *Pseudotsuga menziesii* had barely detectable mitotic spindles using 6-11B-1 (Fig. 2.2a) and no other microtubule array was detectable. However, faint phragmoplasts, although not visible in roots, were seen in similarly prepared *Pseudotsuga menziesii* shoots (not shown). In *Picea glauca*, 6-11B-1 reacted with both spindles and phragmoplasts (Fig. 2.2c), but the staining was much less consistent than with similarly treated sections of *Pinus radiata.* Some spindles and phragmoplasts in *Picea glauca* did not stain with 6-11B-1, however, staining was consistent regardless of the age of the embryo or the part of the embryo observed, except for a slight increase of staining over the provascular tissue (not shown). The antibody to acetylated microtubules reacted with structures distributed in all cells in both *Pseudotsuga menziesii* shoot and *Picea glauca* material (e.g. unlabelled arrow, Fig. 2.2c). These structures, possibly membrane bound organelles, were not stained with the antibody against β-tubulin or in the negative controls lacking exposure to primary antibodies. In another set of experiments, 6-11B-1 stained plastids in *Arabidopsis thaliana* sections (not shown). As observed in *Pinus radiata,* a few cells of *Picea glauca* embryos had very brightly stained cytoplasm when exposed to this antibody. Results using 6-11B-1 in *Pinus contorta* were very similar to those seen with *Pinus radiata,* with staining of spindles, phragmoplasts and cortical microtubules (Fig. 2.2e).

Angiosperms:

All microtubule structures in methacrylate sections of *Allium* roots, and *Helianthus* shoot apices were stained with the antibody to β-tubulin including cortical microtubules and mitotic spindles (Figs. 2.3b, 2.3d). In contrast to this, no detectable microtubule structures were stained using 6-11B-1 (Figs. 2.3a, 2.3c). Similar results were seen using methacrylate sections of *Arabidopsis thaliana* and *Brassica napus* (not shown).

2.3.2 Distribution of acetylated tubulin in isolated cells

Pinus radiata:

Although staining of plant material in methacrylate sections gave a good overall view of the distribution of acetylated tubulin in the context of the whole tissue, the results were sometimes difficult to interpret. For example, when metaphase spindles looked incomplete (Fig. 2.1c), it was unclear if only some bundles of microtubules were acetylated or if only part of the spindle was in the section. In order to derive a more complete understanding of the distribution of acetylated tubulins in different microtubule arrays, a study of whole, isolated cells was undertaken. Figures 2.4 and 2.5 illustrate the localization of acetylated tubulin (2.4a, 2.4c, 2.4e, 2.5a, 2.5c, 2.5e, 2.5g) and β-tubulin (2.4b, 2.4d, 2.4f, 2.5b, 2.5d, 2.5f, 2.5h) during division of *Pinus radiata* root tip cells. The examination of whole cells confirmed that all microtubule arrays within *Pinus sp* root cells contained acetylated tubulin. These included preprophase bands (Figs. 2.4a and 2.4c), prophase spindles (Fig. 2.4c), metaphase spindles (Fig. 2.4e), anaphase spindles (Figs. 2.5a and 2.5c), and phragmoplasts (Figs. 2.5e and 2.5g). Staining of preprophase bands was markedly less extensive with 6-11B-1 than with the antibody against β-tubulin (Figs. 2.4a, 2.4c cf. Figs. 2.4b, 2.4d). Furthermore, a larger number of perinuclear and cytoplasmic microtubules stained with anti β–tubulin than with 6-11B-1 at this stage of cell division. The distribution of microtubules within metaphase and anaphase spindles labelled with 6-11B-1 was similar to that observed when cells were reacted with the antibody to β-tubulin, although acetylated tubulin (Figs. 2.4e, 2.5a, 2.5c) often appeared more granular or discontinuous when compared to β-tubulin (Figs. 2.4f, 2.5b, 2.5d). While the kinetochore fibres in metaphase cells were labelled to a similar extent with both antibodies (Fig. 2.4e cf Fig. 2.4f), anaphase spindles were somewhat less intensely stained with 6-11B-1 (Figs. 2.5a, 2.5c) than that seen with cells at a similar stage stained with the antibody to β-tubulin (Figs. 2.5b, 2.5d).

Strong staining of polar caps with both antibodies was observed during early anaphase (Figs. 2.5a and 2.5b). Interzonal microtubules (Figs. 2.5c, 2.5d) and early phragmoplast microtubules, visible between the two reforming nuclei, were stained with both antibodies (Figs. 2.5e, 2.5f); however, only staining for β-tubulin revealed microtubules radiating from the daughter nuclei into the cytoplasm (Fig. 2.5f). There was strong staining of phragmoplasts with both antibodies (Figs. 2.5g, 2.5h), indicating that a large proportion of the microtubules was acetylated in these structures. Controls receiving only secondary antibodies did not stain (not shown).

In summary, it is clear from the results of staining fixed intact cells of *Pinus radiata* with 6-11B-1 that three microtubule arrays stained more strongly than the others during cell division: the kinetochore microtubules, polar caps at anaphase and phragmoplasts at telophase.

Pinus contorta:

Results of double staining experiments using 6-11B-1 and an antibody to unmodified, tyrosinated tubulin (which labels all microtubules in the cell) confirmed what was seen when cells were stained with antibodies singly in both *Pinus radiata* and *Pinus contorta*. A representative 1 μm section through a group of *Pinus contorta* cells is shown in Fig 2.6a. In this section, both metaphase spindles and a phragmoplast are well stained with 6-11B-1 while microtubules in interphase cells are very lightly stained. Compared to the staining with 6-11B-1, the antibody to tyrosinated tubulin (fig 2.6a') labelled more microtubules in all cells.

Allium cepa:

Even though microtubule structures were well stained with the antibody to unmodified, tyrosinated tubulin (Fig. 2.6b' and 2.6c'), no staining of microtubule structures was seen using 6-11B-1 (Fig. 2.6b and 2.6c).

2.3.3 Acetylated tubulin found in bryophyte cells

In order to determine if acetylated tubulin was present in bryophyte cells, immunoelectron microscopy was used because *Marchantia* cells are so small and autofluorescent that it is difficult to visualize microtubules using fluorescent microscopy. LR White sections without osmium membrane fixation are somewhat difficult to interpret because of the poor preservation of membranes (see section 5.3.4, below), however microtubules are well preserved (arrows in Figs. 2.7a and 2.7b). Acetylated tubulin was found in cortical microtubules (Fig. 2.7a) as well as in other arrays including microtubules surrounding the prophase nucleus (not shown). Similar sections were stained with an antibody to α-tubulin (Fig. 2.7b).

Figure 2.1. Sections of methacrylate embedded *Pinus radiata* root tips. **a** - toluidine blue staining. **b** - section stained with anti-β-tubulin antibody. **c,d,e** - sections stained with 6-11B-1, a monoclonal antibody specific for acetylated tubulin. **b',c',d',e'** - corresponding nuclei stained with DAPI. All micrographs are at the same magnification, bar = 20 μm.

a. Toluidine blue staining of *Pinus radiata* root tip section containing a number of dividing cells.

b. β-tubulin staining in *Pinus radiata* meristem. Prophase (P) and metaphase (M) spindles, and phragmoplasts (Ph) are visible in this section. Note also cortical microtubules (arrowhead).

c. Prominent staining of phragmoplasts (Ph), as well as faint staining of prophase (P), metaphase (M) and anaphase (A) spindles with 6-11B-1.

d. An anaphase spindle (A) and cortical microtubules (arrowhead) stained with 6-11B-1.

e. Anaphase (A) and prophase (P) spindles stained with 6-11B-1 as well as recently divided sister cells with prominent cytoplasmic staining (double arrowhead).

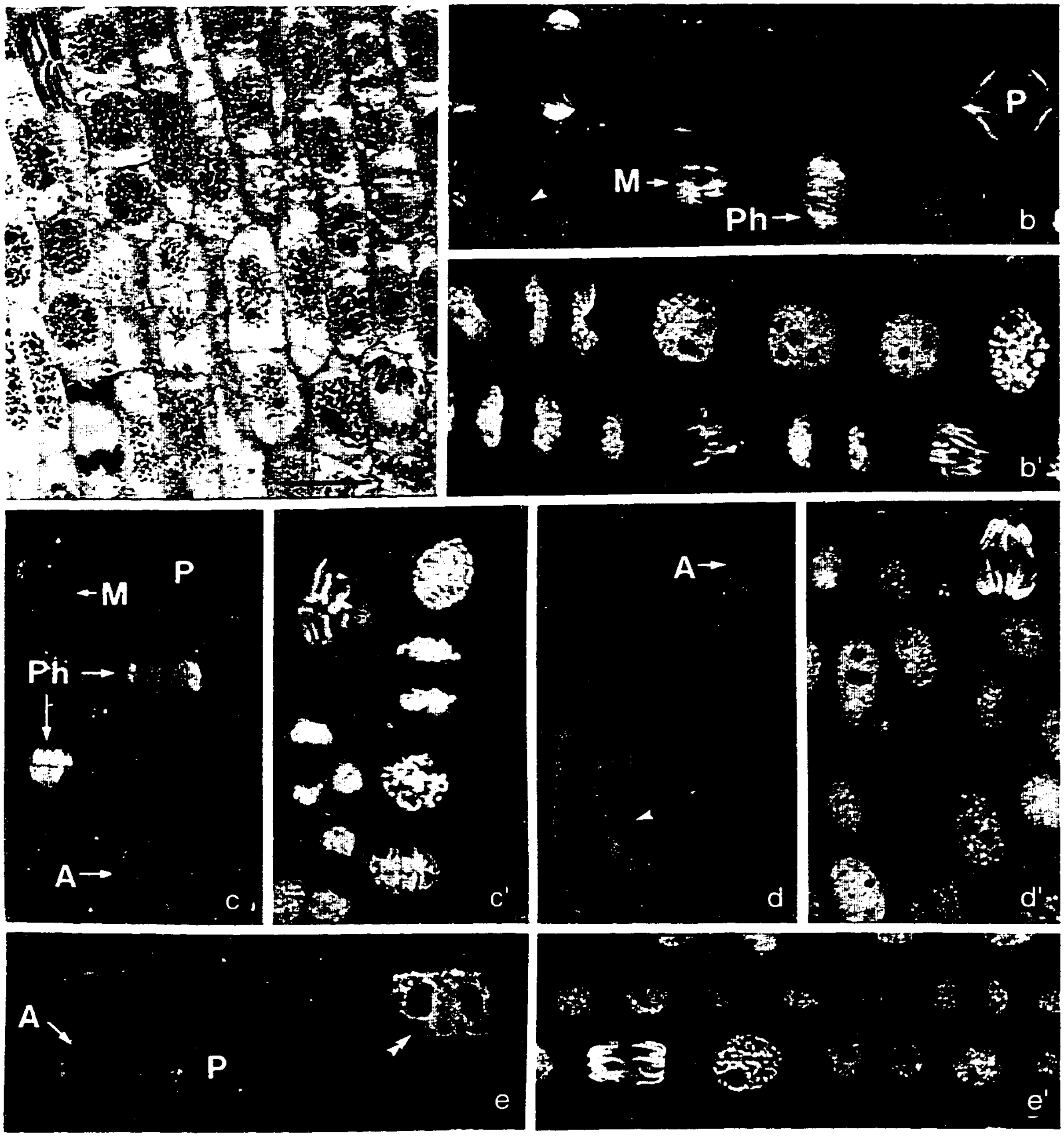

P
M
Ph
b
b'
M
P
Ph
A
c
c'
A
d
d'
A
P
e
e'

Figure 2.2 Sections of methacrylate embedded conifer tissue. **a, b** - *Pseudotsuga menziesii* root tip sections. **c, d** - *Picea glauca* embryo sections. **e, f** - *Pinus contorta* root tip sections. **a, c, e** - sections stained with 6-11B-1, a monoclonal antibody specific for acetylated tubulin. **b, d, f** - sections stained with a monoclonal antibody specific for β-tubulin.. **a' -f'** - corresponding nuclei stained with DAPI. All micrographs are at the same magnification, bar = 20 μm.

a - staining of a *Pseudotsuga menziesii* root tip section. A faint anaphase spindle (A) is visible.

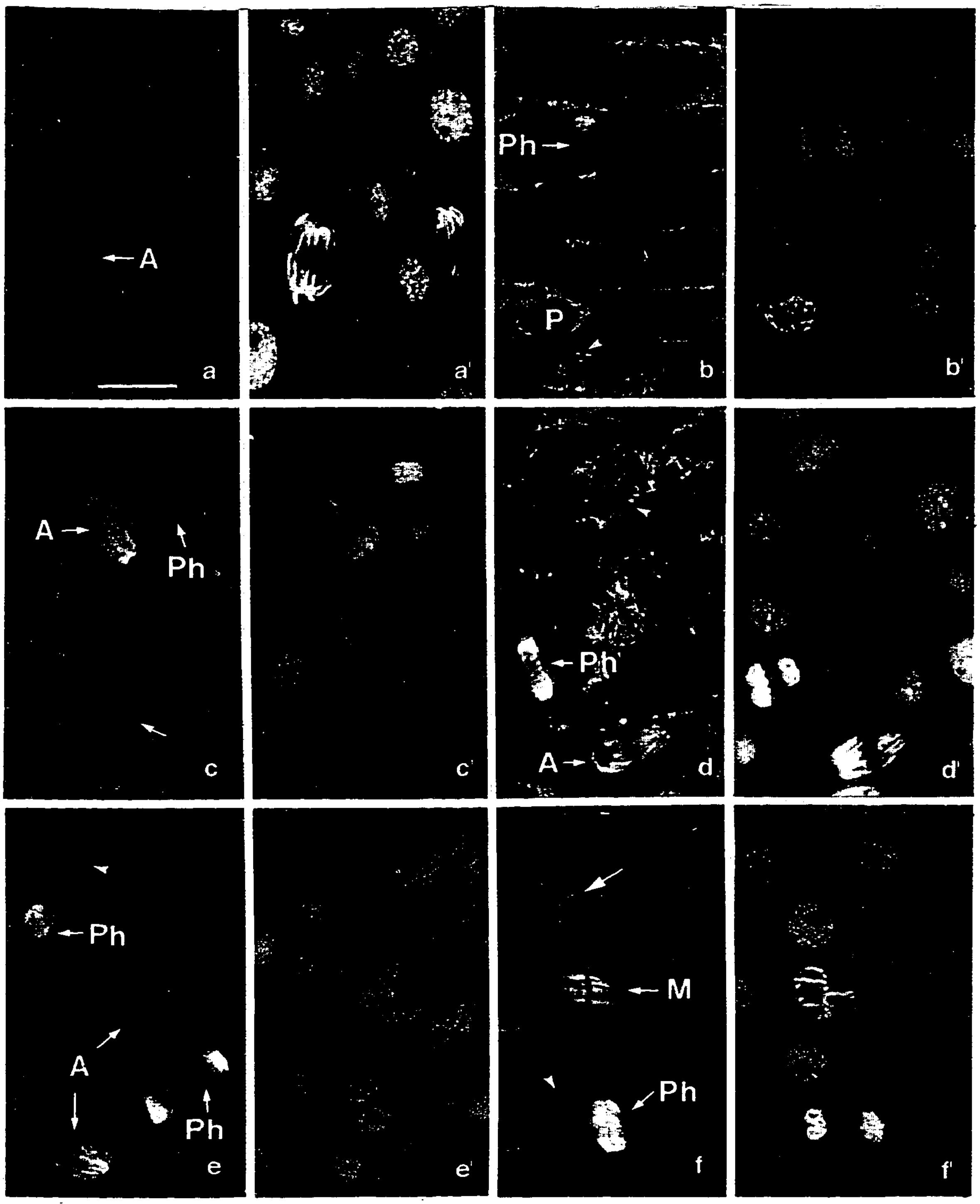
b - β-tubulin staining of a *Pseudotsuga menziesii* root tip section. A prophase spindle (P) and a phragmoplast (Ph) as well as cortical microtubules (arrowhead) are visible.

c - 6-11B-1 staining of a section of cotyledon from an 8 day *Picea glauca* embryo. An anaphase spindle (A) as well as a faint phragmoplast (Ph) are visible. An example of a structure which reacts with 6-11B-1 is marked with an arrow.

d - β-tubulin staining of a *Picea glauca* embryo section. A phragmoplast (Ph), anaphase spindle (A) and cortical microtubules (arrowhead) are clearly visible.

e - 6-11B-1 staining of a *Pinus contorta* root tip section. Anaphase spindles (A), phragmoplasts (Ph) and faintly stained cortical microtubules (arrowhead) are visible.

f - β-tubulin staining of a *Pinus contorta* root tip section. A metaphase spindle (M), phragmoplast (Ph) and preprophase band (arrow) as well as cortical microtubules (arrowhead) are present.


A
a
a'
Ph
P
b
b'
A
Ph
c
c'
Ph
A
d
d'
Ph
A
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e
e'
M
Ph
f
f'

Figure 2.3 Sections of methacrylate embedded angiosperm tissue. **a,b** - *Allium cepa* root tip sections. **c, d** - *Helianthus annuus* L. shoot apex sections. **a, c** - sections stained with 6-11B-1, a monoclonal antibody specific for acetylated tubulin. **b, d** - sections stained with a monoclonal antibody specific for β-tubulin. **a' -d'** - corresponding nuclei stained with DAPI
All micrographs are at the same magnification, bar = 20 μm.

a - 6-11B-1 staining of an *Allium cepa* root tip section. No microtubules detected.

b - β-tubulin staining of an *Allium cepa* root tip section. A metaphase spindle (M) and a phragmoplast (Ph) as well as cortical microtubules (arrowhead) are visible.

c - 6-11B-1 staining of a *Helianthus annuus* L. shoot apex section. No microtubules detected in either cortical or mitotic arrays. An arrow marks a metaphase plate in **c'**, but there is no corresponding metaphase spindle in **c**.

d - β-tubulin staining of a *Helianthus annuus* L. shoot apex section. A metaphase spindle (arrow) and cortical microtubules (arrowhead) are clearly visible.

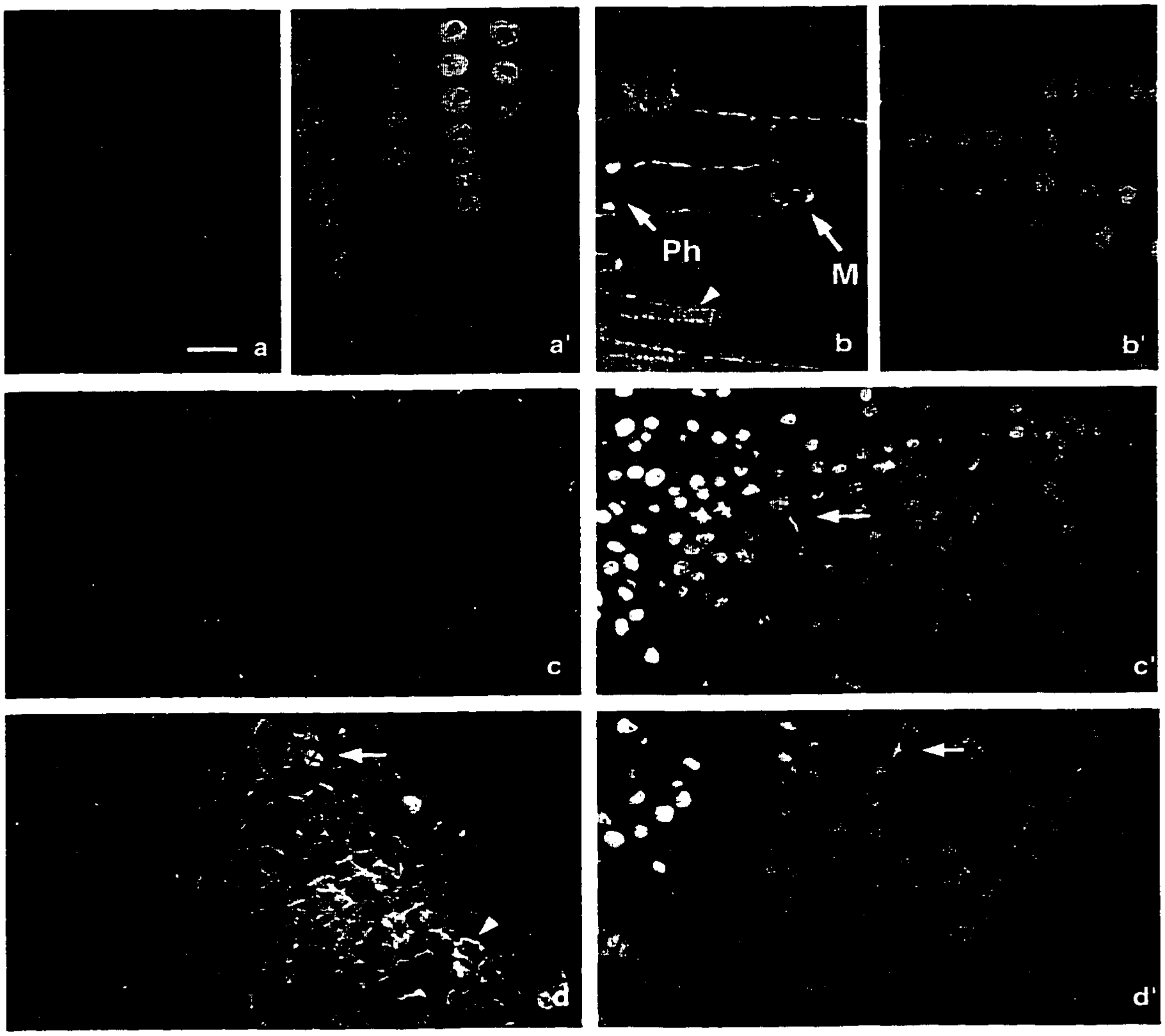

a
a'
Ph
M
b
b'
c
c'
d
d'

Figure 2.4. Preprophase to metaphase in isolated cells from root tip squashes of *Pinus radiata.* **a, c, e** - cells stained with 6-11B-1. **b, d, f** - cells stained with a monoclonal antibody specific to β-tubulin. **a'-f'** - DAPI stained nuclei. All micrographs are at the same magnification, bar = 10 μm.

a - 6-11B-1 staining of preprophase cell with preprophase band and perinuclear microtubules visible.

b - β-tubulin staining of a preprophase cell; preprophase band and perinuclear microtubules are visible.

c - 6-11B-1 staining of an early prophase cell. Note the prophase spindle forming (arrow), and remnants of the preprophase band (arrowhead).

d - β-tubulin staining of a preprophase- early prophase cell. Note focused microtubules at one pole (arrow).

e - 6-11B-1 staining of a cell in metaphase. Note the barrel shaped spindle of kinetochore microtubules.

f - β-tubulin staining of a cell in metaphase.

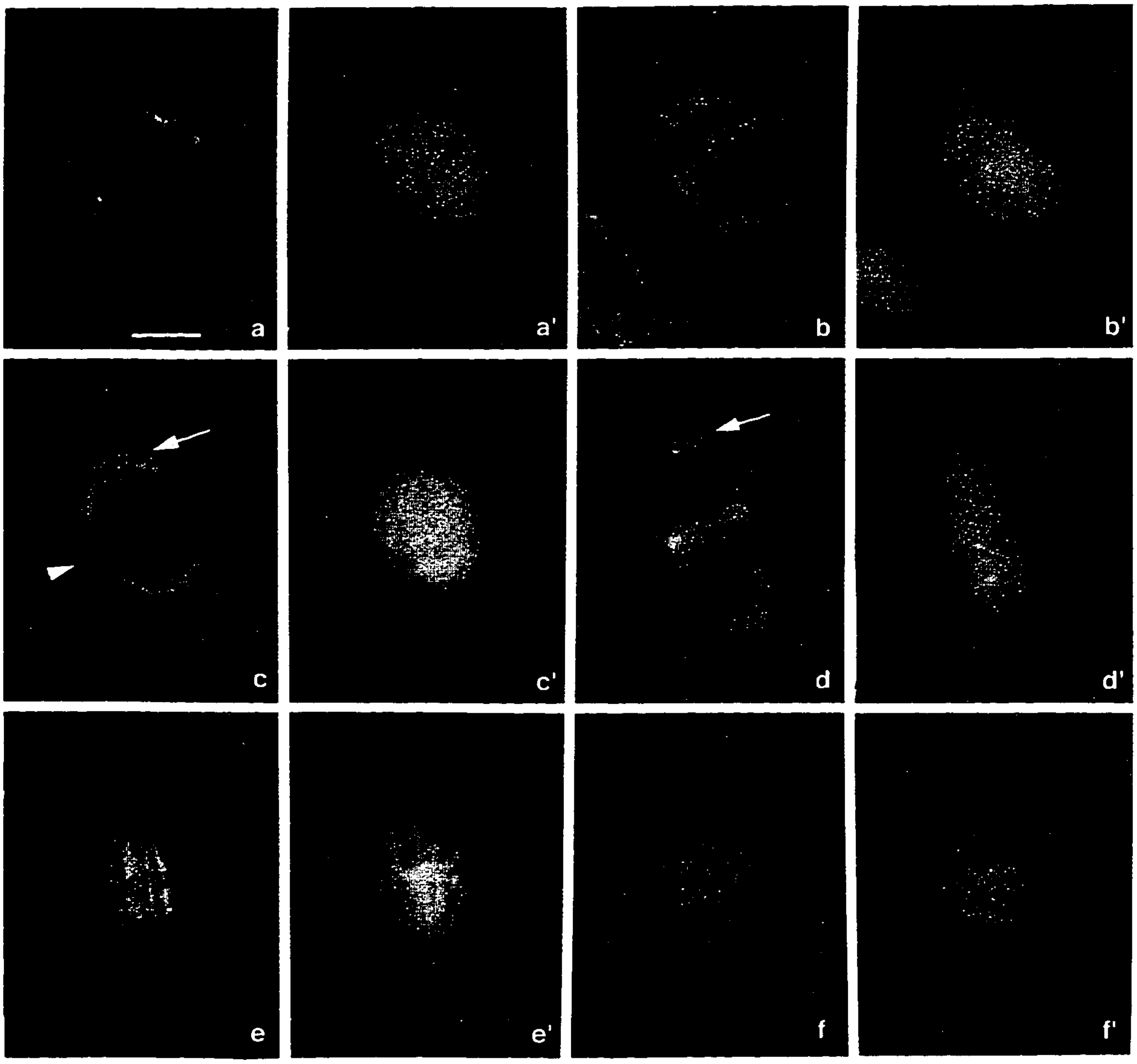

a
a'
b
b'
c
c'
d
d'
e
e'
f
f'

Figure 2.5. Anaphase to telophase in isolated cells from root tip squashes of *Pinus radiata*. **a, c, e, g** - cells stained with 6-11B-1, a monoclonal antibody specific for acetylated tubulin. **b, d, f, h** - cells stained with a monoclonal antibody specific to β-tubulin. **a'-h'** - DAPI stained nuclei. All micrographs are at the same magnification, bar = 10 μm.

a - 6-11B-1 staining of a cell in anaphase, showing strongly stained polar caps.

b - β-tubulin staining of a cell in anaphase, showing strongly stained polar caps.

c - 6-11B-1 staining of a cell in late anaphase when polar microtubules are no longer visible.

d - β-tubulin staining of a cell in anaphase; polar microtubules less focused than in **b**.

e - 6-11B-1 staining of early telophase cell with interzonal microtubules visible.

f - β-tubulin staining of early telophase cell with interzonal microtubules as well as a number of microtubules radiating from all sides of the daughter nuclei (arrow).

g -. 6-11B-1 staining of a telophase cell with uniformly labelled phragmoplast.

h - β-tubulin staining of a telophase cell with phragmoplast.

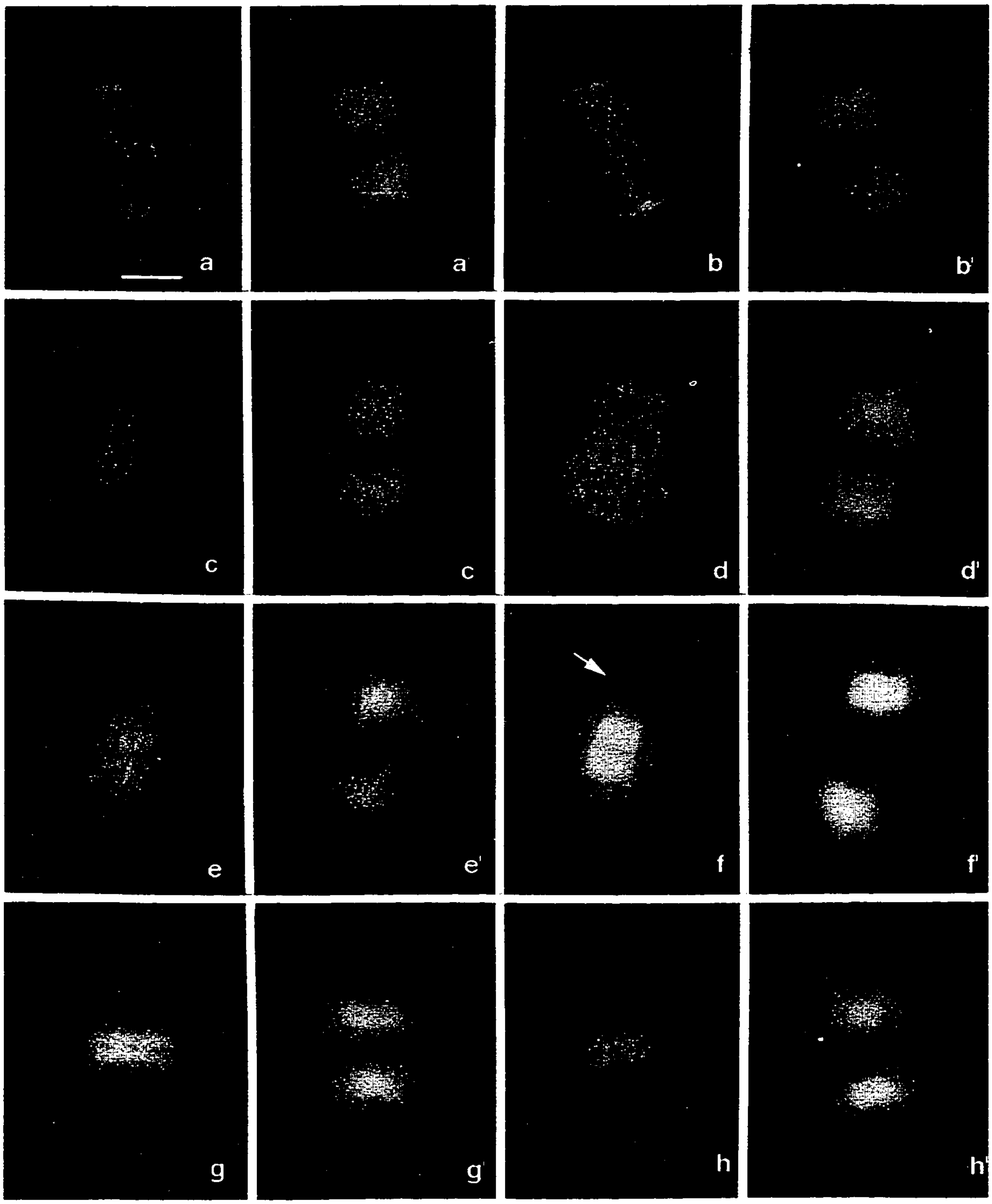

a
a'
b
b'
c
c'
d
d'
e
e'
f
f'
g
g'
h
h'

Figure 2.6. Root tip cells of *Pinus contorta* (**a**) and *Allium cepa* (**b**, **c**) double stained with antibodies to acetylated tubulin and tyrosinated tubulin. One μm optical sections are photographed using confocal microscopy. **a**, **b**, **c** - cells stained with 6-11B-1. **a'**, **b'**, **c'** -the same cells stained with a polyclonal antibody specific to tyrosinated tubulin. **a''**, **b''**, **c''** - corresponding nuclei stained with propidium iodide. All micrographs are at the same magnification, bar = 10 μm.

a - 6-11B-1 staining of a group of *Pinus contorta* root tip cells. A cell with a phramoplast (Ph) and another cell with a metaphase spindle (M) are visible.

a'- The same group of cells with microtubules visualized with a polyclonal antibody to unmodified (tyrosinated) α-tubulin. The phragmoplast (Ph) and spindle (M) visible in **a** are visible here. Note that more microtubules are visible using this antibody.

b - 6-11B-1 staining of an *Allium cepa* metaphase cell. Only non-specific background visible

.

b' - The same cell stained with a polyclonal antibody to tyrosinated α-tubulin.

c - 6-11B-1 staining of a group of *Allium cepa* root tip cells. No microtubules are visible.

c'- The same group of cells with microtubules visualized with a polyclonal antibody to unmodified (tyrosinated) α-tubulin.A cell with a phramoplast is visible.

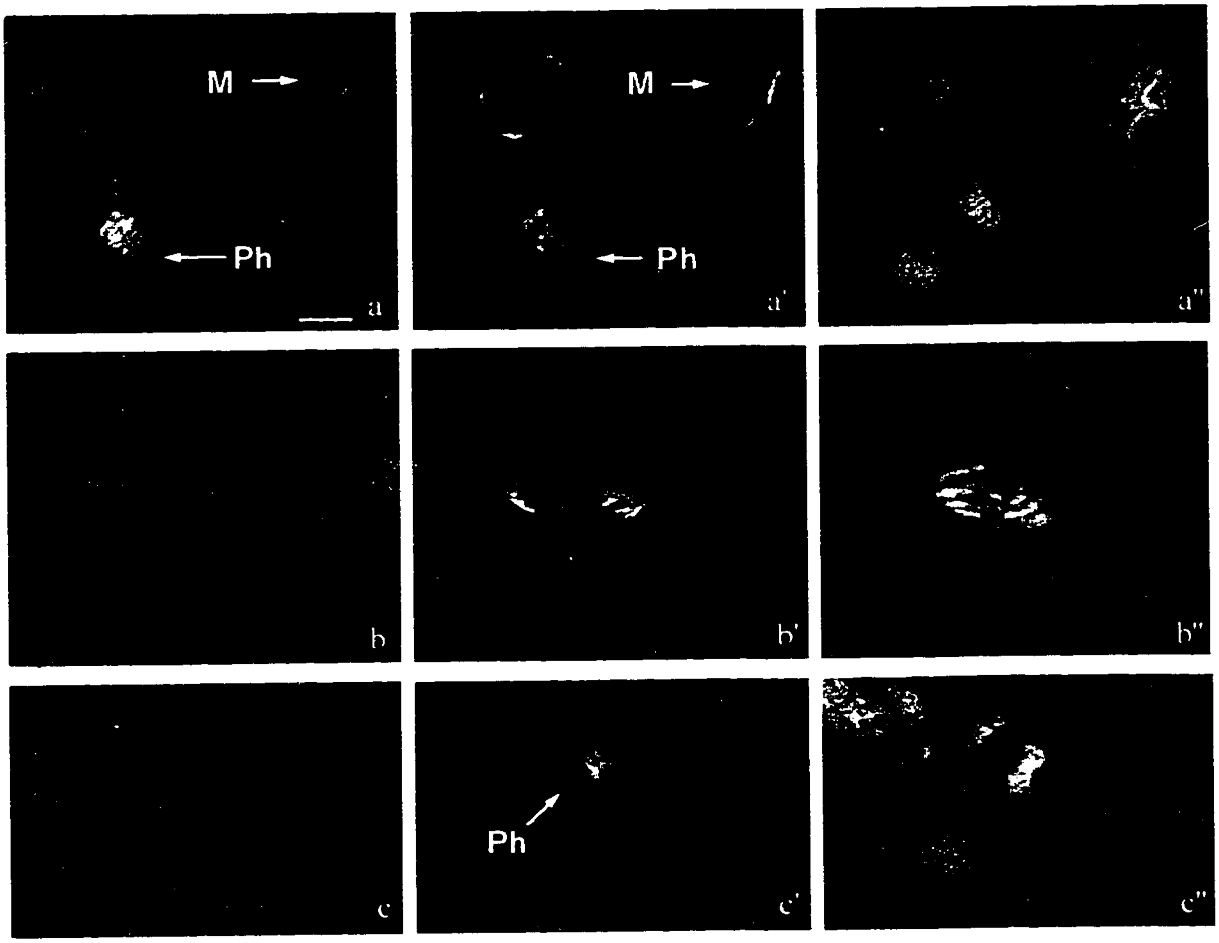
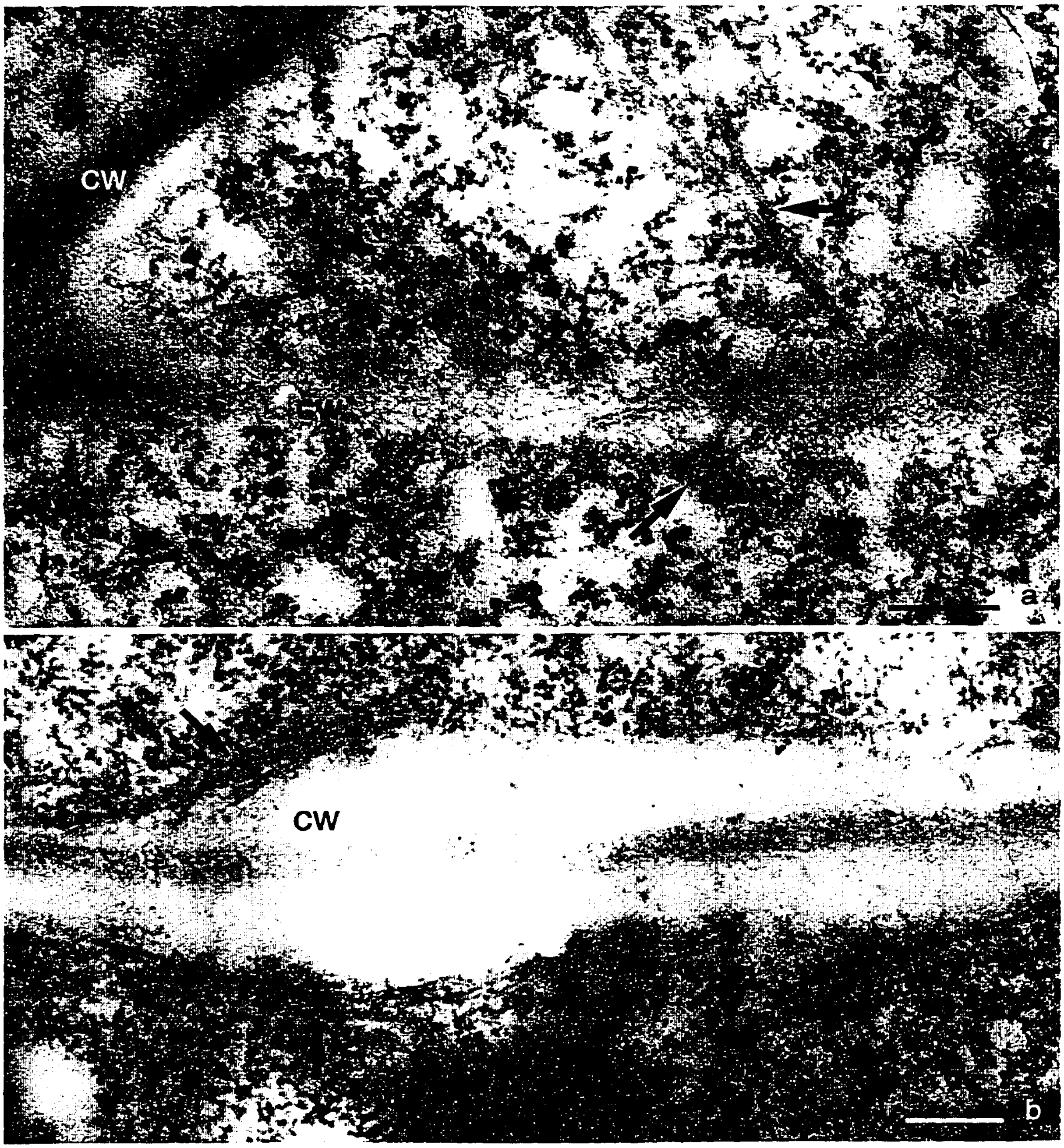

M
Ph
a
M
Ph
a'
a''
b
b'
b''
c
Ph
c'
c''

Figure 2.7. Immunogold labelling of LR White embedded *Marchantia polymorpha* tissue. **a**. tissue labelled with 6-11B-1. **b**. tissue labelled with an antibody to α-tubulin. cw indicates the cell wall, arrows point to microtubules. Bars = 0.2 µm.

a. 6-11B-1 staining of microtubules.

b. α-tubulin staining of cortical micotubules.


CW
a
CW
b

2.4 Discussion

Acetylated tubulins were first discovered in stable microtubule arrays, notably axonemes, basal bodies and centrioles, but subsequent work revealed that other, more dynamic microtubule structures, could also contain acetylated tubulin (Piperno et al. 1987; LeDizet and Piperno 1991; MacRae 1992). The question remains whether there is a correlaton between microtubule stability and acetylation that could be useful in understanding posttranslational modification and the control of microtubule dynamics.

Is there a relationship between acetylation levels and microtuble stability in plant cells? There are a few reports of acetylated microtubules in higher plants (Kerr and Carter 1988; Åström 1992; Smertenko et al. 1997) and some recent work on the dynamic properties of plant microtubules (Hush et al. 1994; Yuan et al. 1994, 1995; Hepler and Hush 1996). Unfortunately, studies of microtubule dynamics and acetylated microtubule distribution have not been performed on the same plant cells although both types of studies have used angiosperm cells. Microinjection experiments using *Tradescantia* stamen hair cells have demonstrated that plant microtubules are very dymanic with turnover rates as high or higher than those of microtubules in animal cells (Hush et al. 1994). Fluorescence redistribution after photobleaching (FRAP) indicates that some arrays are more labile than others (Hush et al. 1994; Yuan et al. 1994, 1995; Hepler and Hush 1996). Based on the relative stabilities of microtubule arrays determined from FRAP studies on *Tradescantia* stamen hairs (Hush et al. 1994) we would predict that mitotic spindles, having the highest turnover rates, would have the lowest proportion of acetylated tubulin compared to other structures. In contrast, preprophase bands, phragmoplasts and cortical arrays, having a lower turnover rate, should have a greater proportion of acetylated tubulin, and consequently more staining with 6-11B-1. These are exactly the results reported in rye (*Secale cereale* L) and tobacco cultured cells where mitotic spindles did not stain or stained only at the poles using 6-11B-1, while preprophase bands, phragmoplasts and cortical microtubules did stain, although noticeably less intensely than with an antibody to total tubulin (Kerr and Carter 1988; Smertenko et al. 1997).

Our results show no detectable acetylation in mitotic microtubule structures at all in the angiosperm cells examined. This may be the result of whole plant tissues having different microtubule dynamics than cultured cells or pollen tubes. Or, the angiosperm species that we used may have different levels of acetyltransferase, deacetylase and inhibitor, or even faster microtubule dynamics than tobacco and rye. The staining of microtubules in *Marchantia* and of all microtubule arrays, including mitotic spindles, in the conifer cells we studied contrasts with the limited distribution in angiosperm cells. This could reflect slower microtubule kinetics generally, different relative microtubule turnover rates in these two evolutionarily distinct groups, or variations in the quantity or activity of the modifying enzymes (MacRae 1997).

In the present study, *Pinus radiata* microtubule arrays showing the strongest staining with 6-11B-1 were the kinetochore fibres, from metaphase through anaphase, the polar cap regions at anaphase and the phragmoplast in telophase, although double staining results indicated that in each case not all of the microtubules were labelled at every stage. Such observations are interesting in terms of the composition, function and presumed stability of these microtubules. Kinetochore microtubules are arranged in bundles linking chromosomes to pole regions and a subpopulation of these microtubules is drug and cold stable (Lambert et al. 1991). It is thought that the kinetochore bundles are composed in part from 'older', more stable prophase spindle microtubules captured by kinetochores after nuclear envelope breakdown (Hepler and Hush 1996). Kinetochore fibres are also selectively stained with 6-11B-1 in crane fly spermatogenic cells (Wilson and Forer 1989).

Strong staining with 6-11B-1 at the spindle poles of conifer cells during anaphase may indicate that these are a stable population of microtubules. Higher plants lack a centrosome, which in most animal cells provides a focus for polar and kinetochore microtubules. However, many higher plants, including conifers (Fowke, unpublished), contain a distinct aggregation of endoplasmic reticulum at spindle poles during anaphase

(Hawes et al. 1981). This aggregation perhaps functions as a focus for microtubules. In this situation, attachment of the spindle to the endoplasmic reticulum would stabilize the microtubules, leading to stronger staining with 6-11B-1, presumably because tubulin acetylase has a longer time to function (MacRae 1997). The microtubules frequently observed radiating from the polar cap at this stage of conifer cell division (Fig. 2.5f) did not stain with 6-11B-1, indicating that they were labile. A similar phenomenon was observed at early telophase, where microtubules radiating from the reforming nuclei were detected by the anti β-tubulin antibody but not with 6-11B-1. These microtubules presumably are newly formed and therefore the tubulin acetyltransferase has not had time to act on them.

A subset of microtubules comprising the phragmoplast may be a relatively stable array during cytokinesis. The phragmoplast initial, a cylinder of microtubules formed in late anaphase between separated chromosomes, is thought to consist of polar spindle microtubules (Hepler and Hush 1996; Staehelin and Hepler 1996). Polymerization at the plus ends of microtubules in this relatively rigid scaffold, or interactions between antiparallel microtubules, may assist in the separation of nuclei at telophase (Baskin and Cande 1990). These relatively stable microtubules are augmented by microtubules nucleated at the reforming nuclear envelopes (Baskin and Cande 1990). In this context, it is interesting that microtubules of the midbody, which like the phragmoplast initial is formed from remnants of the interzonal overlapping anaphase spindle microtubules, are acetylated in some mammalian cells (Piperno et al. 1987; Schatten et al. 1988; LeDizet and Piperno 1991), *Artemia* (MacRae et al. 1991), *Xenopus* (Chu and Klymkowsky 1989) and insect spermatogenic cells (Wilson and Forer 1989).

Clearly, microtubule arrays showing a high degree of acetylation in conifers, such as kinetochore fibres, the anaphase polar cap and the phragmoplast, correspond to relatively stable components of the mitotic and cytokinetic apparatus. In contrast, staining of the preprophase band with 6-11B-1 was generally weak and may reflect a more labile microtubule arrangement. Indeed, a recent study using taxol on wheat (*Triticum aestivum*) root cells indicated that preprophase bands are assembled from newly polymerized microtubules, not from 'older' cortical microtubules translocated to this array (Panteris et al. 1995).

The presence of the epitope recognized by 6-11B-1 in conifer and bryophyte cells reported in this study indicates that these plants have the enzymes involved in the acetylation and deacetylation of α-tubulins. Two features observed in the 6-11B-1 staining may indicate how the modifying enzymes work: some arrays have a granular appearance; certain recently divided cells have a high level of cytoplasmic staining. The granular appearance of the microtubule bundles at metaphase and anaphase using 6-11B-1 probably represents discontinuous stretches of acetylation, indicating that the acetylation occurs at discrete sites. This observation is consistent with the results of studies on *Chlamydomonas* acetyltransferase (Maruta et al. 1986). However, this discontinuity could be the result of antibody-reactive sites hidden by microtubule associated proteins (MAPs) or other cell components. Discontinuous or punctate staining of microtubules with 6-11B-1 has been observed in mammalian cultured cells (Bulinski et al. 1988). Strong cytoplasmic staining in recently divided cells in root tips of *Pinus radiata* and embryos of *Picea glauca* may reflect depolymerized but not yet deacetylated tubulin from the degenerating, highly acetylated phragmoplasts. If so, it would provide further evidence that deacetylation occurs in the cytoplasmic tubulin pool, and not on microtubules just before depolymerization (Piperno et al. 1987).

This is the first report of acetylated tubulin in microtubules of gymnosperms and bryophyte cells. It distinguishes variations in the level of acetylation amongst the different conifer microtubule arrays observed during cell division. The strong staining of certain arrays with 6-11B-1 likely reflects the higher degree of stability of their microtubules. Further studies of acetylated tubulin in a variety of species will assist in our understanding of the correlation between stability and acetylation, perhaps contribute to the mapping of stabilized microtubules, and help determine the role acetylation plays in microtubule function.

2.5 References

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Chapter 3. Detyrosination, a posttranslational modification at the C-terminus of α-tubulin

3.1 Introduction

Detyrosination of α-tubulin and the reverse process, tyrosination, were first discovered more than twenty years ago (reviewed in Pratt et al. 1987). Although most of the research concerning this modification cycle since then has used cultured vertebrate cells, detyrosinated tubulin has been found in other types of cells including those of invertebrates and protists. Detyrosination, like acetylation (Chapter 2) is a posttranslational modification of α-tubulin usually associated with stable microtubules. Like acetylation, its function is not yet understood.

Unlike acetylation, but like all other posttranslational modifications studied to date, detyrosination changes the carboxy (C) terminus of α-tubulin, a portion of tubulin that displays considerable variations among different genetically coded α-tubulin isoforms and the part believed to mediate interactions with other cell components (Sullivan 1988; Burns 1991). Most α-tubulins have the sequence glu-gly-glu-glu-tyr at their C-terminus (Little and Seehaus 1988), but the final tyrosine residue can be removed by an α-tubulin specific carboxypeptidase. This detyrosination of the original tubulin (tyr-tubulin) makes it detyrosinated tubulin (or glu-tubulin) with the terminal amino acid now glutamine. The reverse process (tyrosination) that replaces the tyrosine is performed by the enzyme tubulin tyrosine ligase (MacRae 1997). The same α-tubulin can be detyrosinated and then retyrosinated repeatedly. Because the carboxypeptidase attaches and modifies α-tubulin in polymerized microtubules, detyrosination is essentially a post-polymerizational modification (Bulinski and Gundersen 1991). The tyrosine ligase preferentially modifies α-tubulin in dimers, quickly creating a homogeneously tyrosinated tubulin pool after microtubules have depolymerised (Bulinski and Gundersen 1991). Because the enzymes act preferentially on different forms of α-tubulin, a heterogeneous population of microtubules can be created from a homogeneous population of protomers (Bulinski and Gundersen 1991). Electron microscopy studies using immunogold labelling have revealed that those microtubules which undergo these modifications often contain both types of tubulin – glu and tyr– along their length, although there can be microtubules greatly enriched in one form or another (Sullivan 1988).

When this modification cycle was first described, it was believed that all α-tubulin originally had a terminal tyrosine residue, but later it was discovered that the primary translation product of some α-tubulins such as those encoded by the mouse mα4 and the human hα4 genes, ended in gly-glu-glu, (Sullivan 1988; Cambray-Deakin and Burgoyne 1990) and that tubulin could enter the cycle from either side (Sullivan 1988).

The enzymes responsible for detyrosination and tyrosination have properties that contribute to our understanding of this enigmatic process. The tubulin carboxypeptidase has not yet been purified, but some information is available (reviewed in MacRae 1997). Tubulin carboxypeptidases are distinct from other carboxypeptidases. For example, the *Artemia* tubulin carboxypeptidase is not inhibited by inhibitors of carboxypeptidase A (Xiang and MacRae 1995). In rat brain extract, the carboxypeptidase-microtubule association is regulated by the phosphorylation state of the enzyme or another associated protein (Sironi et al. 1997). Both ATP and phosphatase inhibitors decrease the affinity of the enzyme to microtubules, but the unreactive AMP-PCP does not have this effect (Sironi et al. 1997). In contrast to tubulin carboxypeptidase, tubulin tyrosine ligase is comparatively well understood. The porcine version of tubulin tyrosine ligase has been sequenced and characterized (Ersfeld et al. 1993; MacRae 1997). This enzyme forms a tight complex with the α-β dimer and reattaches tyrosine in a process that requires ATP. The ligase portion is highly conserved at least in mammalian sequences (Ersfeld et al. 1993). The preferred substrate of tubulin tyrosine ligase is at least 12 residues ending with gly-glu-glu but it will accept gly-asp-glu. Because one of the requirements for its binding is that the second last amino acid must be acidic, it will not recognize α-tubulin ending in gly-glu. It does accept polyglutamylated tubulin readily even though this posttranslational modification greatly alters the C terminus (Rüdiger et al. 1994).

Some α-tubulin does not participate in the detyrosination / retyrosination cycle because it has a C terminus that is not recognised by the modifying enzymes. This can be the consequence of a further posttranslational modification as is the case with Δ2-tubulin, or because the original gene product is divergent itself. Δ2-tubulin is the result of a related posttranslational modification in which both the terminal tyrosine and penultimate glutamine have been removed. It was first discovered in brain tissue where it is the major variant of α-tubulin (Paturie-Lafanechere et al. 1994). Because the tubulin tyrosine ligase does not recognize a gly-glu C-terminus as a substrate, it is also called non-tyrosinatable or non substrate α-tubulin (Paturie-Lafanechere et al. 1994; reviewed in MacRae 1997). The enzyme responsible for this modification has not been characterized; in fact it is not known whether Δ2-tubulin is produced by long exposure to the same carboxypeptidase that is responsible for detyrosination, or (more likely) whether an as yet uncharacterised special dipeptidase cleaves both amino acids at once (MacRae 1997). Δ2-tubulin is found in other cells as well, including fibroblasts, where it is confined to stable structures such as centrioles or primary cilia, sea urchin sperm flagella and sea urchin embryo cilia during development (Paturie-Lafanechere et al. 1994). It is absent in some animal cells such as muscle cells (Alonso et al. 1993). Δ2-tubulin is believed not to participate in the tyrosination / detyrosination cycle and this modification is believed to be non-reversible (MacRae 1997).

In spite of the fact that most α-tubulins have a C-terminal sequence of glu-gly-glu-glu (+/-tyr), some divergent isotypes have been found. These include the highly divergent chicken testis α-tubulin (Pratt et al. 1987), α4 in *Drosophila* ending in asp-glu-phe (Warn et al. 1990), and the α-tubulin of *Giardia lamblia* that ends in asp-ala-tyr (Weber et al. 1997). These isoforms are not substrates for the modifying enzymes and therefore do not participate in the tyrosination / detyrosination cycle.

3.1.1 Detyrosination in animal cells

The distribution of detyrosinated tubulin in the microtubules of animal cells has been relatively well studied. The accumulation and analysis of examples of its location in many cell types is a first step in understanding its function. In axons, detyrosination demarcates stable microtubules. One group found distinctly stable domains along axonal microtubules enriched in glu tubulin next to other, more labile sections that were enriched in tyr tubulin (Baas and Black 1990). In later studies, this group (Baas and Ahmad 1992) found that after nocodazole treatment depolymerised the tyr domains, the stable glu microtubules were nucleating sites for microtubules (Li and Black 1996). In cultured PC-12 cells, which are used as models for microtubule involvement in the formation of neuronal processes, each growing neurite becomes progressively more enriched in glu microtubules as it becomes longer. This seems to be an entirely local event with no corresponding detyrosination in other parts of the cell (Bulinski and Gundersen 1991).

In studies using other mammalian cells, detyrosination seems to mark microtubules that are stabilised in the early stages of cellular morphogenesis (Bulinski and Gundersen 1991). For example, in the rat muscle cell line L-6, proliferating non-differentiated cells have low levels of detyrosinated tubulin, but when the cells are switched to differentiation media, detyrosination increases dramatically, very early in the differentiating process (Gundersen et al. 1989; Bulinski and Gundersen 1991). In proliferating fibroblasts and epithelial cells, a small subset of microtubules are very stable, persisting through interphase (16 hr) as compared to most that have half lives of 5-10 minutes (Gurland and Gundersen 1993). These very stable microtubules are enriched in glu tubulin (Bulinski and Gundersen 1991). When fibroblasts migrate into a "wound" produced by scraping some of the cells from a monolayer of these cells, they immediately acquire an additional array of glu microtubules oriented towards the direction of migration (Bulinski and Gundersen 1991). These authors suggest that these stabilised microtubules may serve as specialized tracks for transport of cellular (perhaps membrane) materials required for the migration.

In some animal cells there is clear evidence that detyrosinated microtubules preferentially interact with other cellular components. They are associated with the Golgi complex in rat pituitary tumour cells (GH3 cells), mouse pituitary tumour cells (AtT20/D), monkey kidney cells (BS-C-1 cells), and in mouse L929 cells (Skoufias et al. 1990; Thyberg and Moskalewski 1993). In frog lens epithelial cells, microtubules rich in both acetylated and detyrosinated tubulin form a basket around the nucleus, while tyr microtubules run from the centrosome to the cell periphery (Prescott et al. 1991). An intriguing set of experiments indicate that in polarized, migrating 3T3 fibroblasts, detyrosinated microtubules interact with intermediate filaments (IFs). Injecting these cells with antibodies to detyrosinated tubulin resulted in the collapse of the IF array, but injecting with antibodies to tyrosinated tubulin did not result in this collapse (Gurland and Gundersen 1995). In these experiments, it was clear that IFs are not stabilising the glu microtubules, because the microtubules did not collapse with the IF array (Gurland and Gundersen 1995). The authors suggest that IFs bind to the C-terminus of α-tubulin and this binding is probably stronger with glu tubulin (Gurland and Gundersen 1995). Another example of apparent interactions between detyrosinated microtubules and other cytoskeletal fibre systems was found in studies of the later stages of muscle development in rat neonatal hearts (Webster 1997).

Even though detyrosinated tubulin is found in a particular microtubule structure in one species, it may not be present in that structure in another species. For example, cold adapted Atlantic cod melanophore microtubules are extensively detyrosinated (Nilsson et al. 1996) while black tetra melanophores have little detyrosinated tubulin (Rodionov et al. 1994). Fish brain microtubules can be almost completely detyrosinated in the fresh water fish, *Oncorhynchus mykiss*; while in the marine fish *Labrus berggylta, Zoarces viviparus*, and *Gadus morhua*, brain microtubules consist of a mixture of tyr and glu microtubules (Modig et al. 1994). Interestingly, there were some differences in the way that calcium or colchicine affected assembly in these different populations of fish brain microtubules, but this did not correlate to either acetylation or detyrosination levels (Modig et al. 1994). Other cod microtubules are highly detyrosinated but this is not responsible directly for their cold-stability (Rutberg et al. 1996). In another cell type, erythrocytes, microtubules can be almost completely detyrosinated (in turkey erythrocytes) (Rüdiger and Weber 1993) or completely tyrosinated (in toad erythrocytes) (Spiegel et al. 1991).

Besides the specific location that glu microtubules have in many vertebrate cells, there is other evidence that this modification is highly regulated. For example, in the cerebellum of hypothyroid rats there is a five day delay in the detyrosination of microtubules compared to control rats. Furthermore, the overall activity of the tubulin carboxypeptidase is reduced to about half of normal. This indicates that thyroid hormones are essential for the induction of tubulin carboxypeptidase (Poddar and Sarkar 1993). Another example is the complete and rapid detyrosination of murine macrophage microtubules in response to cell stimulation with phorbol esters. This had no effect on microtubule stability, however, because these newly modified microtubules are not more stable to nocodazole-induced depolymerisation. When nocodazole treated microtubules repolymerised in this study, they were initially tyrosinated even in the presence of phorbol esters (Robinson and Vandre 1995) demonstrating that the tubulin tyrosine ligase functions normally in this situation.

Detyrosination is not limited to microtubules in vertebrate cells. Most microtubule arrays, including spindles and midbodies, in developing larvae of the invertebrate *Artemia* contain detyrosinated tubulin (MacRae et al. 1991; Xiang and MacRae 1995). It also has been reported in insects - *Drosophila* - but in this case it does not appear until the late stages of development and especially during neurone differentiation (Warn et al. 1990). There is an elaborate segregation of a number of posttranslational variants in different parts of the axonemal microtubules of sea urchin sperm flagella. The "A" tubules consist of 95% unmodified tyrosinated α-tubulin, while in "B" tubules, α-tubulin is 65% detyrosinated and both α- and β-tubulins are 40-45% polyglycylated (Multigner et al. 1996).

Protists have a number of different combinations of tyrosination states in their microtubules. Microtubules in some protists such as *Trypanosoma brucei* have both detyrosinated and tyrosinated as well as other posttranslational forms (Schneider et al. 1997). The most ancient eukaryote, *Giardia lamblia*, although it has a number of posttranslational modifications in its microtubules, has a divergent α-tubulin C-terminus and is not detyrosinated (Weber et al. 1997). There is no tyr tubulin in *Trichomonas vaginalis* microtubules but the researchers of this study did not test for detyrosinated tubulin (Delgado-Viscogliosi et al. 1996).

There have been few reports about posttranslational modifications in fungi. There is no detyrosination of α-tubulin in the fission yeast, *Schizosaccharomyces pombe*, even though both isoforms terminate in glu-glu-tyr. This is perhaps a reflection of the fact that these organisms do not have a population of stable microtubules (Alf and Hyams 1991).

3.1.2 Detyrosination in plants

Very few reports exist about the presence of detyrosinated tubulin in plant cells. One study on the presence of posttranslational modifications in the diatom *Thalassiosira fluviatilis* found no detyrosinated tubulin using either immunofluorescence microscopy or Western blots (Machell et al. 1995). It appears that only one group has explored this area in lower plant cells. Hoffman and Vaughn found tyrosinated tubulin in spermatogeneous cells of *Ceratopteris richardii*, in distributions similar to that for total α-tubulin, but they did not report if there were tests with antibodies against glu microtubules. The staining with antibodies to tyrosinated tubulin did not change when cells were treated with taxol (Hoffman and Vaughn 1995a, b).

Two studies looking for tyrosinated tubulin in *Nicotiana tabacum* pollen tubes found tyrosinated tubulin but reported different relative amounts. Åström (1992) found strong staining with YL1/2 (which recognises only tyr tubulin) in both taxol treated and untreated pollen tubes in similar distributions as that for α-tubulin regardless of tyrosination state. Another group (Del-Casino et al. 1993) found the distribution of tyr tubulin punctate and not the same as for antibodies for either α- or β-tubulin. Neither study tested for glu tubulin directly.

There have been two reports of detyrosinated tubulin in higher plant cells. One study demonstrating detyrosination of α-tubulin in plant cells used an indirect method comparing antibody staining of Western blots (Duckett and Lloyd 1994). The researchers were working with dwarf pea seedlings to see if detyrosination could be correlated with the change in the orientation of microtubules in the internode region of dwarf plants exposed to gibberellic acid (GA_3). This plant growth regulator or hormone induces microtubules to become transverse in relation to the plant axis and at the same time promotes stem elongation. The dwarf pea plants have extremely low levels of endogenous GA_3. This study examined the relative staining intensity of dots representing the different isoforms of α-tubulin on 2-D immunoblots with two different antibodies; YOL1/34, which recognizes both detyrosinated and tyrosinated tubulin, and YL1/2, which recognizes only tyrosinated α-tubulin (Duckett and Lloyd 1994). When a specific dot was stained with YOL 1/34 but not with YL1/2 it was concluded that this isoform represented detyrosinated tubulin. The authors were forced to use this indirect method because the antibodies that recognized glu tubulin in animal studies did not recognize it in this plant tissue, so they could not just treat the tissue or blot with an antibody to glu tubulin directly (Duckett and Lloyd 1994). This method was used to detect the change in these tubulin isotypes in dwarf pea stem cells before and after treatment with GA_3. It was suspected that there might be some change in tubulin isotypes with GA_3 treatment because it seemed to cause a dramatic shift in microtubule orientation (from longitudinal to transverse) and increased internodal length. One α-tubulin isotype –α1– was detected by both antibodies in untreated dwarf plants, but only by YOL1/34 (which recognizes both tyr and glu tubulin) in GA_3 treated cells, indicating that this isotype had become entirely detyrosinated during GA_3 treatment. Another isotype $-\alpha_2-$ had dramatically reduced YL1/2 staining, but no decrease in YOL1/34 staining after treatment with GA_3, indicating a similar level of detyrosination. Remarkably, in tall pea stems, both α_1 and α_2 tubulin could be detected only with the YOL1/34 antibody, suggesting that these α-tubulin isotypes are highly detyrosinated in plants with normal levels of GA_3 (Duckett and Lloyd 1994).

The second study demonstrated the presence of glu, tyr and Δ2-tubulin as well as other posttranslational modifications in fixed cells and immunoblots of cultured tobacco cells (*Nicotiana tabacum* L.) (Smertenko et al. 1997). By using 2-D blots, tyrosinated, acetylated and polyglutamylated tubulin were found in a number of α-tubulin isoforms and the antibodies to detyrosinated and Δ2-tubulin recognized only one isoform each. In the intracellular localisation immunofluorescence experiments, the antibody against tyr tubulin heavily stained all microtubule arrays as expected. Glu tubulin and Δ2-tubulin staining was found in all microtubule arrays in all stages of the cell cycle, but only sporadic dot-like staining of cortical and spindle microtubules by either antibody was seen. In contrast to the study of Duckett and Lloyd (1994), these researchers were able to use Gundersen's anti-glu (which has been used in animal studies), and Paturle-Lafanechère's anti-Δ2-tubulin antibodies in both types of experiments (Smertenko et al. 1997). Even though the immunofluorescence portion of these experiments indicated a very limited distribution for these modified forms of α-tubulin, the results suggest that the modifying enzymes may be present in plant cells and justifies further investigation.

3.1.3 Detyrosination and stability

Like acetylation, detyrosination does not directly contribute to microtubule stabilisation (MacRae 1997), but it may act as a "useful indicator" of the level of stability in a particular microtubule population (Duckett and Lloyd 1994). Microtubules enriched in glu tubulin are strikingly more stable to microtubule destabilizing drugs and dilution in extracted cells (Baas and Black 1990; Bulinski and Gundersen 1991). Glu microtubules are longer lived *in vivo* and do not incorporate derivatized or endogenous tubulin (Gurland and Gundersen 1995). However, there is evidence that this stability is related to processes in the cell that are in turn very sensitive to the phosphorylation state of certain cellular components. For example, in a very interesting study (Gurland and Gundersen 1993), phosphatase inhibitors added to NIH 3T3 fibroblasts and TC-7 epithelial cells selectively affected glu microtubules. Tyr microtubules experienced a slight reduction in numbers compared to a complete depolymerisation of glu microtubules. A later study using only TC-7 cells determined that this complete depolymerisation of glu microtubules occurred within 20-30 minutes after phosphatase inhibitor treatment. These glu microtubules persist for 16 hours in untreated TC-7 (Gurland and Gundersen 1995). This shows that stable and labile microtubules are affected differently by protein phosphorylation. Remarkably, kinase activators or inhibitors had no effect on stable microtubules (Gurland and Gundersen 1993). We can speculate that phosphorylation of some cytoskeletal component destabilises these usually stable micotubules because a phosphorylation cascade occurs during the onset of mitosis when even these stable microtubules must become dynamic.

Many studies have indicated that detyrosination is not directly responsible for any associated stability. In *in vitro* studies, microtubules enriched in glu and tyr tubulin have similar instability parameters (Idriss et al. 1991), and polymerise equivalently (Raybin and Flavin 1977; Kumar and Flavin 1982). In the macrophage study mentioned above (Robinson and Vandre 1995), microtubules that are completely detyrosinated in response to cell stimulation with phorbol esters, are not more stable to nocodazole-induced depolymerisation.

3.1.4 Possible functions

As is the case with other posttranslational modifications, there is continuing interest in determining the function of the detyrosination / tyrosination cycle. The distinctive and specific association of glu microtubules with certain cellular components like the Golgi complex (Skoufias et al. 1990) and intermediate filaments (Gurland and Gundersen 1995) indicates that detyrosination may mediate this association. It may be that detyrosination marks those microtubules destined for organelle interaction thus distinguishing them from those that participate in the polymerisation / depolymerisation dynamics (Gurland and Gundersen 1995). In fact, a functional relationship between the Golgi complex in GH3-rat pituitary tumour cells (and other mammalian cultured cells) and detyrosination of α-tubulin may exist. The authors of one study suggest that detyrosination may facilitate the binding of a MAP 2 -like protein or a 58 kDa MAP associated with Golgi or alternatively that these MAPs may stabilize microtubules that may then be detyrosinated (Skoufias et al. 1990). This proposed functional relationship between Golgi and glu tubulin is in apparent contradiction with earlier reports that injecting cells with YL1/2 (an antibody to tyr tubulin) disrupted Golgi. The authors point out that in the earlier studies, the anti-tyr antibodies could have prevented detyrosination, or that the microtubules, even though they were enriched with glu-tubulin, had sufficient tyr tubulin to be affected by anti-tyr tubulin antibodies (Skoufias et al. 1990). Dynein may also interact preferentially with glu microtubules. Studies looking at the effect of ortho-vanadate on mitotic spindles in *Xenopus* tadpole heart cells indicated that dynein colocalizes with glu microtubules (Winkelhaus and Hauser 1997), but this association could have been merely coincidental.

Because increased levels of both acetylated and glu microtubules are highest in cells undergoing morphogenic events, these posttranslational changes may be important in morphogenic processes linked to maturation and differentiation of certain cells (Bulinski and Gundersen 1991; Ersfeld et al. 1993; Gurland and Gundersen 1995). The elaboration of glu microtubules often is one of the first events in the cytoplasmic remodelling that occurs during myogenesis (Gundersen et al. 1989). Glu microtubules appeared before acetylated tubulin and before myosin in these cells (Gundersen et al. 1989).

Because so little is known about the presence of detyrosinated tubulin in plant cells, this study was undertaken in a number of angiosperm and gymnosperm species to determine if and where detyrosinated microtubules are located in plant cells. By using antibodies raised against tyr-, glu- and Δ2-tubulin, no evidence of either modified form in plant cells was found, whereas tyr-tubulin was present in all microtubule arrays. The glu- and Δ2-tubulin epitopes were, however, created *in vitro* by treating both sections and fixed whole cells with carboxypeptidase A. This confirmed that negative results on untreated cells were due to a real lack of these modifications in the plant cells and not to procedural problems.

3.2 Materials and Methods

3.2.1 Plant material

Pinus radiata, *Pinus contorta*, *Pseudotsuga menziesii* and *Allium cepa* root tips were prepared as described in Chapter 2 (2.2.1.) One mm segments of young siliques from *Arabidopsis thaliana* plants were fixed and prepared as described for root tips above.

3.2.2 Preparation of methacrylate sections and Preparation of root tip cells

Methacrylate sections and root tip cells were prepared as described in Chapter 2 (2.2.2 and 2.2.3, respectively.)

3.2.3 Immunofluorescence staining of methacrylate sections and isolated cells

Immunofluorescence staining was performed as described in Chapter 2 (2.2.4) with the following exceptions: a subset of slides in each study containing sections or cells were treated with cold methanol for 10 minutes at -20°C during initial experiments. This treatment did not affect staining for any antibodies used in this study. The antibodies used were affinity purified polyclonal antibodies or serum, anti Y, anti E and NTT that recognize tyr microtubules, glu microtubules and non-tyrosinatable (Δ2-tubulin) microtubules, respectively, and were a gift from Dr T MacRae, Dalhousie University, Halifax (described in Xiang and MacRae 1995). These antibodies were diluted 1/500 or 1/1000 in PBSBT (PBSB with 0.5% Tween-20). The monoclonal antibody against β–tubulin described in Chapter 2 was diluted as previously described. Staining was performed as described in Chapter 2, although sometimes incubation times were reduced to 1 hour at 37°C with no incubation at 4°C with no apparent change in intensity of staining.

Sections treated with carboxypeptidase A were exposed to the enzyme after incubation with acetone to remove the resin, but before preblocking and exposing to antibodies. Cells were exposed to carboxypeptidase A (Sigma C9268), after the cells were fixed and extracted with Tx-100, but before exposing to antibodies. Carboxypeptidase A was used at concentrations ranging from 0.001 units/ml to 0.5 units/ml diluted in MtSB with surprisingly no detectable difference in outcome, although at higher concentrations, the cells and sections lifted from the slide surface to a greater extent. Cells and sections were incubated for 15 minutes at 37°C, then washed with a stream of PBS and then 3 changes of PBS over 20 minutes. Control sections or cells were treated with PBS in place of carboxypeptidase A.

3.3 Results

3.3.1 Distribution of tyrosinated, detyrosinated and non-tyrosinatable tubulin in methacrylate embedded sections of plant tissue

Pinus radiata:

All microtubule arrays were consistently stained using anti-Y, the polyclonal antibody that recognises tyr tubulin, α-tubulin that has retained the terminal tyr residue (Fig. 3.1a and also Fig 2.6a' where anti-Y was used to visualise total tubulin arrays in double staining experiments with 6-11B-1). In contrast, no staining of recognizable microtubule structures were visualized using anti-E, an antibody to detyrosinated tubulin (Fig 3.1b) or anti-Δ 2-tubulin (NTT, Fig 3.1c) even though microtubule structures were highly visible using an antibody to β-tubulin, double labelled in the same sections (e.g.Fig. 3.1b').

Picea glauca somatic embryos:

Results with other conifer tissue were similar to those obtained with *Pinus radiata* root tips. In methacrylate sections of *Picea glauca* somatic embryos, anti-Y stained microtubules (Fig 3.1d), while anti-E (Fig 3.1e) and NTT (Fig 3.1f) did not. All three polyclonal antibodies exhibited noticeably more cytoplasmic staining than the monoclonal anti-β-tubulin antibody (e.g. Fig 3.1b" cf. Fig 3.1a), but microtubules were easily detected over this dull background.

Angiosperm tissue:

Onion root tip microtubules did not react with anti-E or NTT (not shown). However, as was the case for conifer tissue, microtubules did stain with antibodies to tyr-tubulin (Fig 3.2a) and β-tubulin (Fig. 2.3b). Similar results were obtained with other angiosperm species. For example in *Arabidopsis* silique tissue, no microtubule staining was observed with anti-E (Fig 3.2c) but microtubules were stained in neighbouring sections with both anti-Y (Fig 3.2b) and the antibody to β- tubulin (Fig 3.2d).

3.3.2 Distribution of tyrosinated, detyrosinated and non-tyrosinatable tubulin in whole isolated cells

Pinus sp.

Supporting the results using sectioned tissues, anti-Y stained all microtubule arrays in whole isolated pine root tip cells (e.g. Fig 3.3a), whereas no microtubule structures were stained with either antibody to modified forms, anti-E or NTT (Figs.

3.3b, 3.3c), over a range of antibody dilutions (1/50 to 1/1000) and preparation conditions in spite of background cytoplasmic staining. This was further confirmed with immunoelectron microscopy results in which no gold particles were associated with visible microtubules when sections were stained using these antibodies (not shown). Typical β-tubulin staining showing intense staining of all microtubule arrays is included in Figure 3.3d for comparison.

3.3.3 Creation of the epitope recognized by antibodies to detyrosinated and Δ2-tubulin by carboxypeptidase A treatment

Plant material in methacrylate sections:

Figure 3.4 shows the results of staining with anti-E and NTT after sections had been treated with a commercially available enzyme–carboxypeptidase A. Onion sections treated with 0.05 units/ml carboxypeptidase A, and subsequently stained using anti-E, the antibody to detyrosinated tubulin (Fig. 3.4a) exhibit a remarkable similarity to those undigested sections stained earlier with the antibody to tyr-tubulin (Fig. 3.2a) in that all microtubule arrays are now stained. Figure 3.4b shows a neighbouring section in the same experiment treated exactly the same as that in 3.4a except it was exposed to MtSB instead of carboxypeptidase to confirm that anti-E does not stain undigested microtubules. When conifer sections were digested with carboxypeptidase A, microtubules were also recognized by the antibody to detyrosinated tubulin only after digestion with carboxypeptidase A (Fig. 3.4c; compare to Fig 3.1b for results with the same antibody without treatment with carboxypeptidase A). Figure 3.4c illustrates one unwanted consequence of digestion with the enzyme, in that parts of sections and whole cells often lifted from the surface of the slide, making photography difficult.

Not only anti-E but also NTT, the antibody to α-tubulin that is missing both the glu and tyr amino acids from its C- terminus, stained sections treated with carboxypeptidase A. Figure 3.4d illustrates this in a section of Douglas fir tissue, and staining with NTT was observed on carboxypeptidase treated sections of other plant material (not shown).

Whole cells:

The creation of the detyrosinated and Δ2-tubulin epitopes also occurred when whole cells were digested with carboxypeptidase A. Although many cells were lifted from the slide surface leaving behind microtubule "footprints" stained with antibodies to both modified forms (not shown), some whole cells remained and were well stained with the antibody to detyrosinated tubulin. These, like the onion sections noted above, had remarkable similarity to those untreated cells stained with antibodies to tyrosinated or β-tubulin. Figures 3.5a, 3.5b, 3.5c and 3.5d are examples of pine cells in which a number of different microtubule arrays are stained with anti-E after treatment with carboxypeptidase A (0.01 or 0.001units/ml). Figure 3.5e depicts the same epitope created in onion cells; anti-E antibodies stained an anaphase spindle after carboxypeptidase A treatment. Figure 3.5f shows a cell in the same experiment on the same slide, treated with anti-E under the same conditions but without the prior carboxypeptidase treatment, and no microtubule staining. This illustrates that the carboxypeptidase does indeed create the epitope needed for this antibody to recognise microtubules.

Figure 3.1. Methacrylate sections of conifer tissue. **a, b,** and **c**-- *Pinus radiata* root tip sections. **d, e, f**-- *Picea glauca* somatic embryo. **a** and **d**--sections stained using anti Y, an antibody to tyrosinated tubulin. **b** and **e**-sections stained using anti-E, an antibody to detyrosinated tubulin. **c** and **f**– sections stained using NTT, an antibody to Δ2-tubulin. **b"**–the same section shown in **b**, but stained using an antibody to β-tubulin. **a'-f'** DAPI staining of corresponding sections. All micrographs are at the same magnification, bar = 20μm.

a. Phragmoplast (Ph), anaphase (A) and metaphase (M) spindles are stained using anti-Y.

b. No microtubules detected with anti-E. Arrows indicate cells that have phragmoplast and metaphase spindle containing β-tubulin and indicated by Ph and M respectively in b".

c. No microtubules detected with anti-Δ2-tubulin antibodies. Arrows indicate two cells in division with no spindle staining.

d. A phragmoplast (Ph), preprophase band (arrow) and cortical microtubules (arrowheads) are stained with anti-Y.

e. No microtubules detected with anti-E.

f. No microtubules detected with NTT. Arrow indicates a cell in division with no spindle staining.

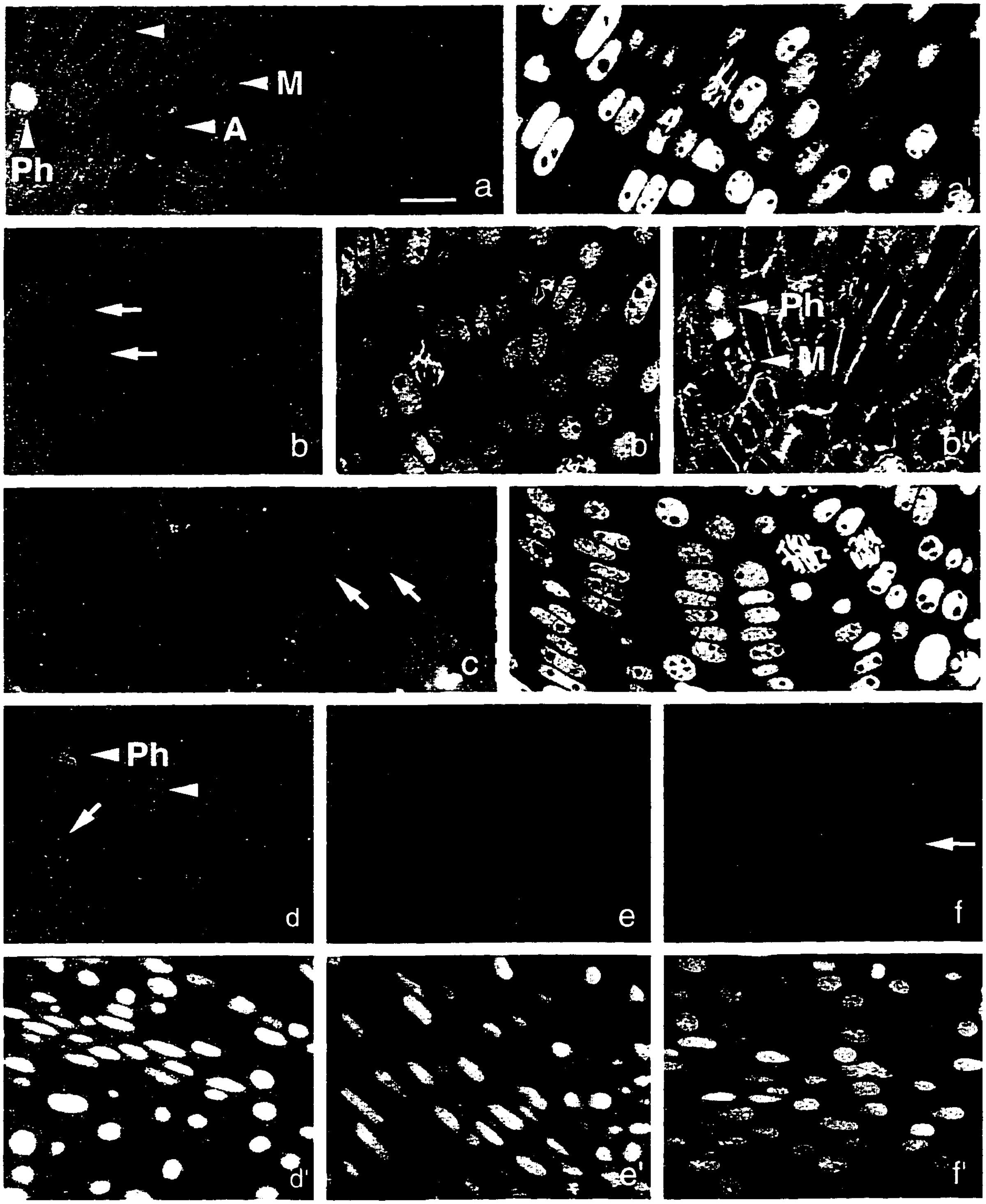

M
A
Ph
a
a'
b
b'
Ph
M
b''
c
Ph
d
e
f
d'
e'
f'

Figure 3.2. Methacrylate sections of angiosperm tissue. **a** – *Allium cepa* root tip section. **b, c,** and **d,** *Arabidopsis thaliana* silique sections. **a** and **b**-sections stained using anti Y, an antibody to tyrosinated tubulin. **c** –section stained using anti-E, an antibody to detyrosinated tubulin. **d** –section stained using an antibody to β-tubulin. **a'-d'** DAPI staining of corresponding sections. All micrographs are at the same magnification, bar = 20μm.

a. All microtubule structures stained with anti-Y in this oblique onion root tip section. Note prominent phragmoplast (Ph), and cortical microtubules (arrowheads).

b. Cortical microtubules (arrowheads) stained with anti-Y.

c. No microtubules detected with anti-E.

d. Cortical microtubules (arrowheads) stained with an antibody to β-tubulin.

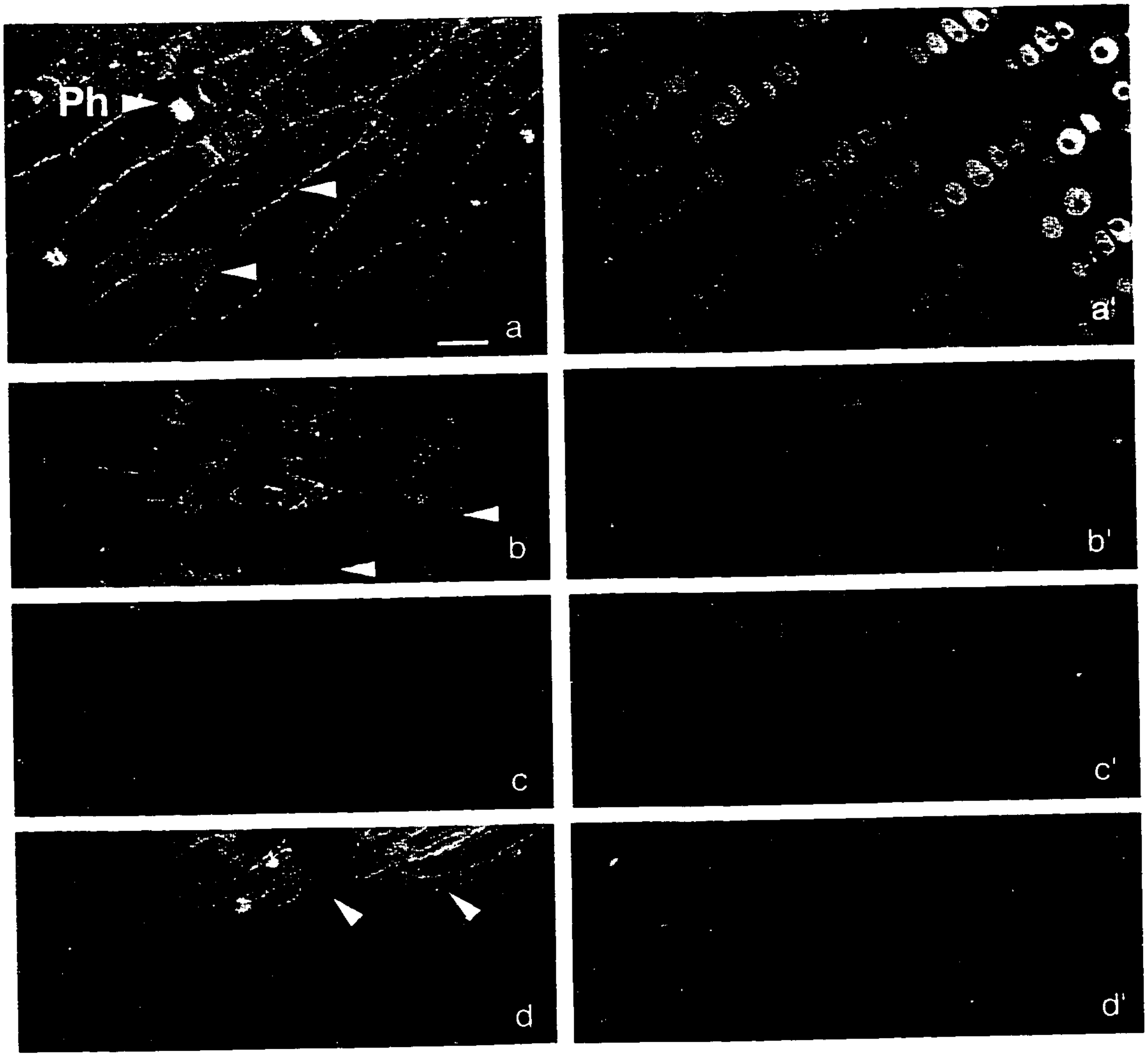

Ph
a
a'
b
b'
c
c'
d
d'

Figure 3.3. Whole isolated *Pinus radiata* root tip cells. **a** – cells stained using anti Y, an antibody to tyrosinated tubulin. **b** – cell stained using anti-E, an antibody to detyrosinated tubulin. **c** – cell stained using NTT, an antibody to Δ2-tubulin. **d** –cells stained using an antibody to β-tubulin. **a'-d'** DAPI staining of corresponding cells. All micrographs are at the same magnification, bar = 10μm.

a. Interzonal spindle microtubules in a cell in late anaphase (A), and a preprophase band (arrow) are both stained with anti-Y.

b. No staining of a spindle microtubules with anti-E.

c. No spindle microtubules detected with NTT.

d. All microtubule arrays stained with an antibody to β-tubulin including phragmoplast (Ph), prophase spindle (P) and preprophase band (arrow).

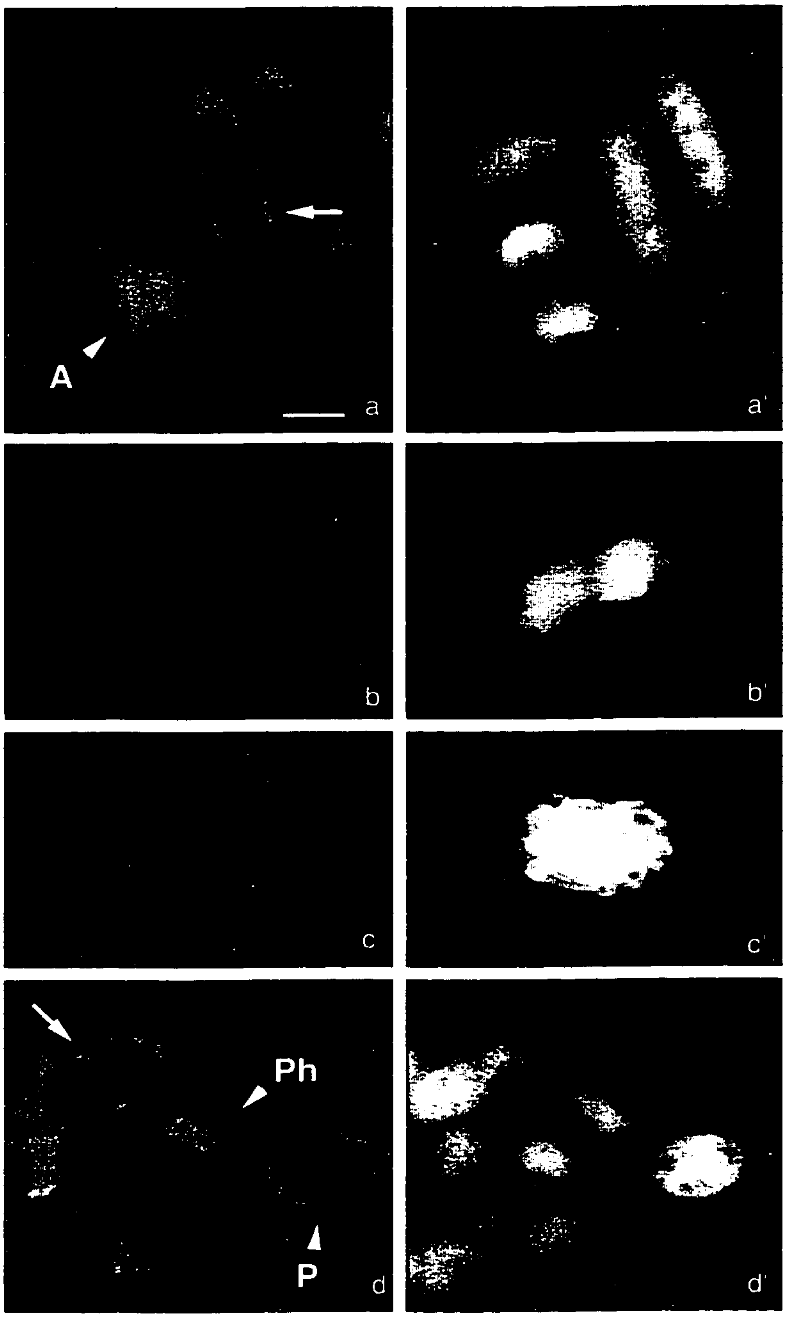

A
a
a'
b
b'
c
c'
Ph
P
d
d'

Figure 3.4. Methacrylate sections treated with carboxypeptidase A before staining. **a** and **b**- *Allium cepa* root tip section. **c** – *Pinus contorta* root tip section. **d** – *Pseudotsuga menziesii* root tip section. **a** and **c** sections stained using anti-E, an antibody to detyrosinated tubulin. **d** – section stained using an antibody to Δ2-tubulin. **a'-d'** DAPI staining of corresponding sections. All micrographs are at the same magnification, bar = 20 μm.

a. All microtubule arrays stained with anti-E after carboxypeptidase A treatment, including phragmoplast (Ph), metaphase spindle (M), and cortical microtubules (arrowheads).

b. No microtubules stained with anti-E without carboxypeptidase A treatment.

c. All microtubule arrays stained with anti-E after carboxypeptidase A treatment, including a phragmoplast (Ph) and a metaphase spindle (M).

d. Microtubules including those in phragmoplasts (Ph) are stained with the antibody to Δ2-tubulin after carboxypeptidase A treatment.

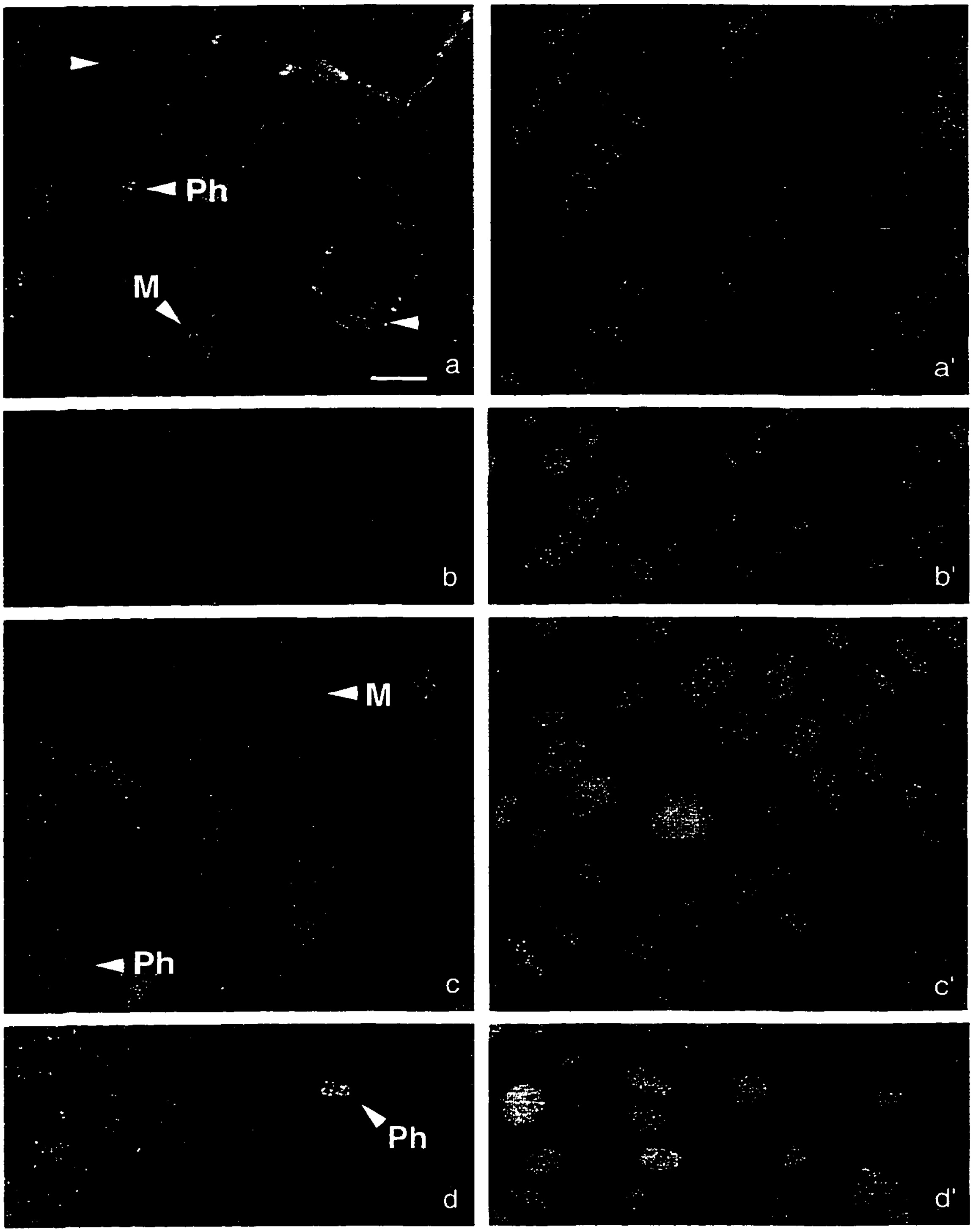

Ph
M
a
a'
b
b'
M
Ph
c
c'
Ph
d
d'

Figure 3.5. Isolated, whole root tip cells stained with anti-E, an antibody to detyrosinated tubulin after carboxypeptidase A treatment. **a -d** *Pinus radiata* isolated root tip cells. **e** and **f** -*Allium cepa* isolated root tip cells. **a'- c'**, **e'**and **f'**–DAPI staining of corresponding cells. All micrographs are at the same magnification, bar = 10 µm.

a. Pine cell with microtubules stained with anti-E after carboxypeptidase treatment showing prominent preprophase band (arrow).

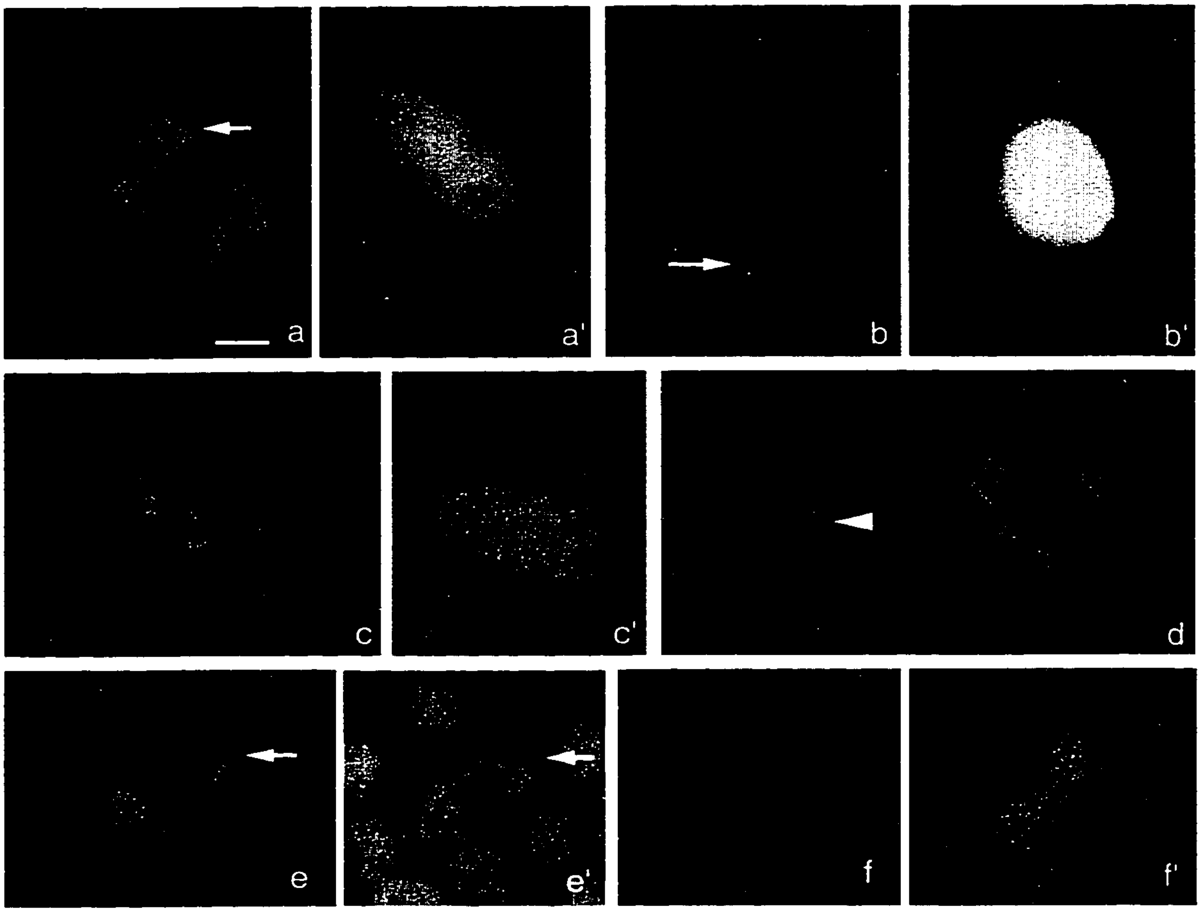
b. Prophase cell with prophase spindle (arrow at one pole) stained with anti-E after carboxypeptidase treatment.

c. Metaphase cell with spindle stained with anti-E after carboxypeptidase treatment.

d. Cortical microtubules stained with anti-E after carboxypeptidase treatment.

e. Onion anaphase cell with spindle microtubules stained with anti-E after carboxypeptidase treatment (arrow indicates one pole).

f. Onion cell in same experiment with no microtubules stained with anti-E without carboxypeptidase treatment.


a
a'
b
b'
c
c'
d
e
e'
f
f'

3.4 Discussion

This study indicates that detyrosinated and Δ2-tubulin are not present in conifer and angiosperm cells. Furthermore, we provide evidence that the microtubules in these cells have abundant tyrosinated tubulin, indicating that this probably unmodified form is the major variant in these cells. The fact that both glu- and Δ2-tubulin are evident after *in vitro* carboxypeptidase treatment indicates that the failure to detect the modified tubulin in untreated sections and cells was not due to problems with maintaining the epitopes throughout sample preparation, nor was it due to problems with the antibodies recognizing the plant versions of modified α-tubulin. The creation of the epitope recognized by antibodies to detyrosinated tubulin or Δ2-tubulin with carboxypeptidase A, an enzyme isolated from bovine pancreas, appears to be a first report in sections or cells. The creation of the detyrosinated epitope on nitrocellulose blots has been reported (Xiang and MacRae 1995).

Our results of the presence of tyrosinated tubulin in all microtubule arrays confirm results of other immunodetection studies in which plant microtubules have been shown to have tyrosinated tubulin (Åström 1992; Hoffman and Vaughn 1995a). Indeed, all six of the α-tubulin genes of *Arabidopsis* code for a tyrosinated form (Kopczak et al. 1992). Our results differ somewhat with two recent reports indicating the presence of glu-tubulin in plant cells (Duckett and Lloyd 1994; Smertenko et al. 1997). It may reflect the fact that sample preparations differed so that in the present study the epitopes were masked and in the *Nicotiana* study they were exposed. Another possibility is that the antibodies we used may not be as sensitive as the ones used by Smertenko et al. (1997). Neither of these explanations is very likely in light of the fact that detyrosinated tubulin was detected in our material when the epitope was artificially created. The fact that both glu- and Δ2-tubulin were found in *Nicotiana* cultured cells but not in our conifer or angiosperm cells may indicate a difference between cultured cells and organized plant tissue, or between tobacco and other species. The discrepancy between our results and Smertenko et al. (1997) may also stem from the fact that their immunofluorescence results indicated that the modified forms of tubulin are present in tobacco cultured cells in very small quantities. In our studies, such small quantities may have gone undetected even if they had been present. However the very small quantities of detyrosinated tubulin is in itself very different from what we would expect given the results from animal studies.

It should be noted that in animal studies, when glu-tubulin is present, it is present in relatively large quantities. As was mentioned in the introduction, even $\Delta 2$-tubulin, for example, is a major variant in some brain tissue although in other cells it is restricted to the centrosome (Paturie-Lafanechere et al. 1994) and in no reports have very small segments of microtubules been the only evidence of the modified forms. There are at least two explanations for the very limited distribution of glu- and $\Delta 2$-tubulin reported by Smertenko et al. (1997). One possible explanation is that the modifying enzymes present in animal cells are also in plant cells, but the tubulin carboxypeptidase is present in a much smaller amount, or is inhibited in some way. Another explanation is that the tubulin in microtubules is being modified by another carboxypeptidase that is present in the tobacco cells and can use tubulin as a substrate, but does not modify it very efficiently. This could explain why both glu-tubulin and $\Delta 2$-tubulin are found in the same cells: they could be the result of a very inefficient carboxypeptidase gradually cleaving terminal amino acids from the carboxy terminus of α-tubulin.

The somewhat indirect evidence for detyrosinated tubulin in elongating internodal regions of GA_3 treated dwarf pea plants (Duckett and Lloyd 1994; and see section 3.1.2) and normal pea plants is both intriguing and puzzling. It is intriguing, because the apparent detyrosination occurs during a process that modifies the microtubule array, and could very well be the result of selective stabilization associated with the effects of GA_3 exposure. It is puzzling because it indicates that the detyrosination is very different from that which occurs in animal cells. For example, this plant detyrosination seems to be divorced from the detyrosination/ retyrosination cycle. In animal cells, tubulin dimers are retyrosinated shortly after glu-tubulin is depolymerised. There is good evidence from studies on living cells that plant microtubules, including pea cortical microtubules (Yuan et al. 1994) have turnover rates at least as rapid as those in animal cells (Hepler and Hush 1996). Even if these internodal microtubules were more stable than usual as a result of GA_3 exposure, it is extremely unlikely that they would not turnover at all. If these plant cells have tubulin tyrosine ligase that functions as it does in animal cells, then there should be tyr-tubulin in the elongating plant internodes. However, no tyr version of α_1 and little tyr- α_2- tubulin was detected in this study (Duckett and Lloyd 1994). A second difference between this apparent detyrosination in plant cells and what occurs in animal cells is that in the pea plants only two out of the three isoforms appeared to be detyrosinated. The major isoform, α_3 seemed to be stained equally by the two antibodies, indicating that it was not detyrosinated to any great extent. The authors do not comment on this unusual aspect of plant detyrosination. It should be noted, however, that the tall plants, which seemed to have only detyrosinated forms of α_1 or α_2, also had detyrosination of α_3 only when they were treated with GA_3. It would be very interesting to have immunolabelling of these plants to map the portions of microtubules that are composed of glu- and tyr- tubulins. Another puzzling aspect of the results of the Duckett and Lloyd (1994) study is that the detyrosinated tubulin was not detected by the anti-glu antibody that was used in animal studies, when the same antibody did detect some glu-tubulin in *Nicotiana* cells (Smertenko et al. 1997) and a similar antibody recognized carboxypeptidase treated plant microtubules in the present study.

There are aspects of both detyrosination and the creation of the Δ2-tubulin that are unique to animal cells, and indicate that they may be restricted to these organisms. (1) Nervous tissue, which is enriched in both glu-tubulin and Δ2-tubulin as well as other posttranslational modifications of tubulin (Laferrière et al. 1997), has a highly specialized cytoskeleton, capable of extensions of up to a metre in length. The microtubules in these cells are also extremely stable compared to other animal microtubules. For example, the average half life of rat axonal microtubules is 2.2 hours, but there are tightly controlled gradations in this stability with certain parts of the cytoskeleton much more stable than others (Laferrière et al. 1997). It is likely that there is an elaborate, possibly overlapping and redundant system of controls regulating the dynamics of this cytoskeleton. Although plants have their own set of elegant cytoskeletal structures, there are probably not ones comparable in complexity to those in neuronal cells. (2) Cellular morphogenesis is completely different in plants and animals, therefore the enrichment of glu in a specialised set of microtubules that are stabilised in the early stages of animal cellular differentiation would probably have no parallels in plant cells. (3) The two other cellular components with which glu microtubules may preferentially interact – the Golgi apparatus and intermediate filaments – are also quite different in animals and plants. The Golgi in animals is usually one complex structure held near the nucleus and centrosome by microtubules, many of which are enriched in glu-tubulin. In plant cells, the Golgi apparatus is usually scattered throughout the cytoplasm as dictyosomes. Intermediate filaments are very poorly documented in plant cells and certainly are not as widely distributed in plant cells as they are in animal cells. In fact many of the structural functions that intermediate filaments have in animal cells are fulfilled by the cell wall in plant cells. In light of these differences between plant and animal cells, it is unlikely that a system of detyrosination / tyrosination would have developed in plant cells in the same fashion as it has in animal cells.

Further studies focussing on different tissues, and a broader survey of plant species, is needed to determine the extent of detyrosination in plant cell microtubules. If this modification is found in other plant cells, it will be necessary to isolate a plant tubulin carboxypeptidase and tubulin tyrosine ligase. Otherwise, the detyrosination may be simply the result of another cellular carboxypeptidase, with the detyrosination of tubulin an accidental, non-functioning side-effect. This may very well be a modification that is a very different process in plant cells than in animal cells. A deeper understanding of these differences will have implications for our understanding of larger questions regarding the overall control of microtubule dynamics.

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Chapter 4. Components of animal MTOCs have novel distributions in plant cells

4.1 Introduction

Microtubules are not permanent structures; they are constantly being assembled, both to replace the microtubules in each pattern or array and to transform one array into another as the cell cycle dictates. Microtubules are assembled by organelles called microtubule organizing centres (MTOCs). These MTOCs promote assembly of microtubules – microtubule *nucleation* – and also place important constraints on the positions of microtubules in cells and the polarity of microtubules in the various microtubule arrays – microtubule *organization* (Oakley 1995; Vaughn and Harper 1998). There is a remarkable morphological diversity of MTOCs in different types of organisms (Oakley 1995). In animal cells, the MTOC is almost always a centrosome that consists of a pair of centrioles surrounded by pericentriolar material, while in fungal cells, a plaque embedded into the nuclear envelope called the spindle pole body (SPB) is usually the MTOC (Balczon 1996). In many fungal species, this elaborate structure can form microtubules both into the nucleus (for an intranuclear mitosis) and into the cytoplasm. In lower plants, the plastid membrane in hornworts, mosses, and lycopsids, and a structure called the polar organizer in hepatics seem to act as MTOCs (Brown and Lemmon 1990, 1992). In spermatogenous tissue of pteridophytes, complicated highly structured MTOC structures arise *de novo* in the formation of multiflagellate motile sperm (Hoffman and Vaughn 1995). Higher plant cells do not have centrioles, and no structured MTOC has been identified, although evidence is strong that the nuclear envelope acts as a microtubule nucleation site during at least part of the cell cycle, and sites on other membranes may have this function (Seagull 1989; Lambert 1993; Stoppin et al. 1996).

It is believed that centrosomes, and presumably all MTOCs, form microtubules by acting as templates at their minus ends, ensuring that a certain number of protofilaments make up each microtubule (Marc 1997). Even though microtubules can assemble spontaneously in high concentrations of tubulin, centrosomes facilitate microtubule formation in cells where the tubulin concentrations are too low for this uncontrolled polymerization (Gunning and Hardham 1982; Vaughn and Harper 1998). A centrosome has a finite number of nucleating sites and this number can change in response to cellular signals (Vaughn and Harper 1998). Even though it is accepted that in the vast majority of animal cells that have centrosomes, they are the site for microtubule nucleation, many questions remain regarding how this process is accomplished and what components are involved. Some resolution to these questions has been made in the last few years with the accumulation of evidence that γ-tubulin is an essential component of microtubule nucleation (Oakley 1995; Zheng et al. 1995).

Even though plant cells assemble microtubule arrays, they do so without a recognizable, discrete microtubule organizing centre (MTOC) like those found in animal and fungal cells. There is even a debate about whether plant cells have an actual MTOC (reviewed in Balczon 1996). It has been suggested, for example, that plant microtubules have specific qualities that allow for the self-assembly of microtubules and microtubule arrays (e.g.Smirnova and Bajer 1998). This idea of microtubule assembly and organization without an MTOC is supported by recent discoveries that brain tubulin can self-assemble into microtubules around chromatin coated beads and then self-organize into very normal looking spindles without centrosomes (Heald et al. 1997; Merdes and Cleveland 1997). The self-assembly appears to be spontaneous, but the organization into spindles appears to depend on microtubule motors, specifically kinesin-like plus end directed motors that sort microtubules into an anti-parallel array, and cytoplasmic dynein that is necessary to form a focused pole (Heald et al. 1997; Merdes and Cleveland 1997). This non-centrosomal microtubule organization also seems to occur *in vivo* in some insect oocytes; the fundamental role of motors in spindle organization may be important in all spindle formation (Balczon 1996). Both the self-assembly of microtubules on chromatin and the organization of spindles by microtubule motors could very well explain some of the observations of microtubule self-organization and chromosome movement in endosperm (Smirnova and Bajer 1998). Other groups have suggested that higher plants have MTOCs but these consist of MTOC components distributed more diffusely and perhaps associated with membranes (Seagull 1989; Lambert 1993; Balczon 1996). For example, there is good evidence that the nuclear envelope acts as a nucleation site in higher plants (Vantard et al. 1990; Schmit et al. 1994; Schmit et al. 1996; Vaughn and Harper 1998). Cortical cytoplasmic regions such as those seen in the water fern *Azolla* and other cortical sites also appear to nucleate microtubules (reviewed in Seagull 1989, Cyr 1994).

It may be more useful to treat microtubule nucleation and polymerization separately from microtubule organization. For example, microtubules may be nucleated and polymerized on γ-tubulin containing complexes, but organized into arrays by a combination of microtubule motors and other microtubule associated proteins (MAPs). This separation of nucleation and organizational functions has recently been supported by results in animal epithelial cells in which microtubules were polymerized at the centrosome, then translocated and docked at another site (Tucker et al. 1998).

One method that has been used to identify plant MTOCs or microtubule nucleating sites is to probe plant cells using antibodies raised against components present in animal and fungal MTOCs (Vaughn and Harper 1998). This approach has been fruitful in finding a number of common components located in MTOCs of animal, fungal, lower plant, and protist cells (Kuriyama 1992; Kalt and Schliwa 1993; Hoffman et al. 1994; Oakley 1995) all of which have identifiable structural MTOCs. We have used well characterized antibodies to three ubiquitous components of animal centrosomes, γ-tubulin, centrin and the antigen recognized by the antibody 6C6, in order to try to identify microtubule nucleating sites in conifer cells. These gymnosperm cells represent a group of higher plants that are evolutionarily distinct from angiosperms, the focus of most of the research on higher plant MTOCs

4.1.1 Gamma tubulin (γ-tubulin)

Evidence is accumulating that γ-tubulin is an ubiquitous MTOC component and is also essential for *in vivo* microtubule nucleation (e.g.reviews in Kalt and Schliwa 1993; Marc 1997). Oakley and Morris discovered γ-tubulin as a suppressor of a β-tubulin gene mutation in *Aspergillus nidulans* (Oakley 1995). After cloning this gene they discovered that it belonged to a tubulin superfamily as similar to β-tubulin and α-tubulin as they are to each other (Oakley 1995). Since then, γ-tubulin has been found in a large number of different organisms (Oakley 1995). In both fungal and animal cells, it is concentrated at the MTOCs, but a substantial fraction is located in the cytoplasm (Stearns et al. 1991; Oakley 1995; Moudjou et al. 1996). Since its discovery less than 10 years ago, versions of the γ-tubulin gene have been cloned from animal, fungal and plant species. γ-tubulin is well conserved between kingdoms. In fact, when human γ-tubulin was expressed in yeast cells lacking functional endogenous γ-tubulin, it localized to the SPB and was able to nucleate normal looking, functioning spindles and cytoplasmic microtubules (Horio and Oakley 1994; Oakley 1995). This conservation of γ-tubulin structure and function probably indicates that other MTOC proteins that would interact with γ-tubulin are likewise functionally conserved (Horio and Oakley 1994; Oakley 1995).

The discovery of γ-tubulin has facilitated a number of productive research approaches to determine the structure of MTOCs. The finding that γ-tubulin is located at the minus end of microtubules (Li and Joshi 1995; Moritz et al. 1995) and is part of a ring structure with a particular ratio of components (Zheng et al. 1995) has helped to devise a model for a microtubule nucleating structure (Zheng et al. 1995). This model proposes that 13 molecules of γ-tubulin are held in a helical template (γ-TuRC or γ-somes) that caps the minus ends of microtubules (Zheng et al. 1995; Marc 1997). It also accommodates the variety of forms of MTOCs because the nucleating units can be arranged in a variety of ways: in the pericentriolar material in animals, on the SPB in fungal cells, and presumably on membranes in plant cells (Marc 1997).

When it was first discovered, most mammalian γ-tubulin was thought to be bound to the cytoskeleton (Joshi et al. 1992), but now it is believed that a sizeable proportion of γ-somes exist in soluble complexes in the cytoplasm (Stearns and Kirschner 1994; Oakley 1995; Zheng et al. 1995). These γ-somes bind to microtubules *in vitro*, but do not nucleate microtubules (Oakley 1995), indicating that they must be activated in some way. They are probably not active when they are newly synthesized because this would cause chaos in the cytoskeleton. Their activation may involve posttranslational modifications of components, the interaction of MTOC components, or the release of the components from sequestering complexes (Horio and Oakley 1994; Balczon 1996). In spite of uncertainty about how the nucleation process is regulated, γ-tubulin complexed with other proteins appears to be an essential component in animal and fungal cells.

Although the localization of γ-tubulin is crucial to our understanding of microtubule organization in higher plants, there has been remarkably little information about its presence in higher plants. In fact, for many years all reports came from one research group (Liu et al. 1993; McDonald et al. 1993; Liu et al. 1994; Liu et al. 1995), and it was not until recently that these results were confirmed by others (Dráber et al. 1997; Endlé et al. 1997; Marc 1997; Binarova et al. 1998). Although there are some variations, all of these results indicate that the spatial distribution of γ-tubulin in plants is very different from that in animals. Staining with γ-tubulin antibodies is not even restricted to a number of possible plant nucleating sites like the nuclear envelope. Instead, it is more conspicuously associated with microtubule structures. Some reports describe a relatively restricted distribution, limited to the minus ends of spindle microtubules and patches in the phragmoplast (Marc 1997) while others report it in distributions similar to that of α- or β-tubulin (Endlé et al. 1997). Some very convincing studies indicate that γ-tubulin is present along all microtubule arrays except towards the plus ends of microtubules (Liu et al. 1993; Hoffman et al. 1994; Liu et al. 1994, 1995). For example, in fluorescence microscope images of both metaphase spindles and phragmoplasts in cells double stained with antibodies to γ- and β-tubulin, there is a noticeably wider gap in the kinetochore region of metaphase cells, and the cell plate area of the phragmoplast with γ-tubulin antibodies (Liu et al. 1994, 1995). Others have found γ-tubulin at the kinetochores, which are microtubule plus end structures (Dráber et al. 1997; Binarova et al. 1998). γ-tubulin is found both in blepharoplasts, which are structural MTOCs in pteridophyte spermatogenous cells, and along microtubules in other fern cells. This distribution along microtubules was not found at plus ends (Hoffman et al. 1994; Vaughn and Harper 1998). In spite of these inconsistencies, all reports of the distribution of plant γ-tubulin include some microtubule staining.

Microtubule staining by γ-tubulin antibodies has been reported in only a very few cases in animal cells, and then only in very limited distributions. For example, γ-tubulin has been found in mitotic spindles and midbodies in 3T3 cells (Novakova et al. 1996). In the acentriolar mouse embryos, γ-tubulin staining was found at the minus end regions of spindle microtubules; this staining quantitatively decreased as the distance increased from the pole (Gueth-Hallonet et al. 1993). The authors speculated that this staining of the acentriolar spindle microtubules could be either because of a more diffuse distribution of minus ends in the absence of centrioles or due to the presence in these cells of a large pool of soluble γ-tubulin, some of which attached to the spindle microtubules (Gueth-Hallonet et al. 1993). These few examples of microtubule staining in animal cells by γ-tubulin are very unusual; the more usual situation is for the γ-tubulin staining to be restricted to the centrosome (Stearns et al. 1991).

The apparently widespread distribution of γ-tubulin staining along microtubules in plant cells is perplexing; and instead of identifying microtubule nucleating sites in plant cells, it raises new questions about the functions of γ-tubulin in cells in general (Vaughn and Harper 1998). How can the apparent presence of γ-tubulin along microtubules be reconciled with its presumed role in microtubule nucleation? There have been a number of suggestions. Marc (1997) proposed two possibilities for this staining along microtubules: we may be seeing the result of the staggered microtubule minus ends each capped with a γ-tubulin ring complex; or we may be observing γ-somes transported on microtubules. Vaughn and Harper (1998) suggest another possibility – that γ-tubulin is incorporated into the microtubule itself, either by substituting for a β-tubulin in a heterodimer, or by the insertion of a ring of γ-tubulin "thirteenmers" periodically along a plant microtubule (Vaughn and Harper 1998). [The possibility that γ-tubulin can form dimers with other tubulins, in this case β-tubulin, is raised by the finding that the ratio of α- to β-tubulin in surf clam centrioles is not the expected 1:1 but rather 1:4or 5 (Vogel et al. 1997).] Endlé et al. (1997) suggest that microtubule distribution of γ-tubulin may be the result of its activation and redistribution during the cell cycle. This idea that γ-tubulin is distributed along microtubules may indicate a plant solution to γ-tubulin storage and distribution that is distinct from the sequestering of γ-tubulin in animal cells. As mentioned above, there can exist a large soluble cytosolic pool of γ-tubulin in both animal and fungal cells where γ-tubulin is bound with other proteins into large complexes (Oakley 1995; Moudjou et al. 1996; Akashi et al. 1997). Over- or trans-genic expression of γ-tubulin genes seems to have relatively benign consequences in non-mammalian cells. This may indicate that storage, sequestering capacities exist in the recipient cells. When a human γ-tubulin gene was over-expressed in fission yeast cells producing levels 5 times that of normal, the cells survived (Horio and Oakley 1994). Constitutive expression of a maize γ-tubulin gene in tobacco BY-2 cells did not seem to affect microtubule arrays, but the excess γ-tubulin appeared to accumulate in the cell wall (Endlé et al. 1997). Other groups believe that microtubule distribution of γ-tubulin may indicate it has a role in microtubule dynamics (Joshi and Palevitz 1996), in particular, a role in microtubule stabilization especially at the kinetochore poles in plant cells (Dráber et al. 1997). Whatever the explanation, there are aspects to the distribution and perhaps the function of γ-tubulin which seems to be unique to plant cells.

4.1.2 6C6

In 1992, Lambert's lab raised a monoclonal antibody to calf thymus centrosomes that recognized a protein in animal cells that was tightly and specifically associated with the pericentriolar material and also was associated with metaphase kinetochores and the midbody. In plant cells, this antibody, 6C6, stained the nuclear envelope in interphase and telophase cells in a distribution that seemed to coincide with microtubule foci on this membrane (Chevrier et al. 1992). Because the nuclear envelope is believed to be a plant MTOC, this localization of a centrosomal component was very exciting. These researchers speculated that the 6C6 antigen was not only a possible microtubule nucleating factor but also an example of a passenger protein that physically moved to different locations in a cell cycle dependent manner. This was because in mammalian cells it was located in the centrosome, then the kinetochores after the nuclear envelope broke down, and the midbody during cytokinesis (Chevrier et al. 1992). Four years later, they discovered that this antigen was also present in the synaptomnemal complex of Gingko and lily meiotic cells (Schmit et al. 1996). In an earlier study, however, they did not find a similar localization in *Allium* or *Tulbaghia* cells; instead they found 6C6 labelled centromeres or kinetochores during meiosis as well as mitosis (Schmit et al. 1994). One other lab has reported results using this antibody; in this case, it was reported on the apical part of pollen tube plasma membranes, a possible MTOC (Cai et al. 1996).

The antigen recognized by 6C6 has not been characterized; however in Western blots, 6C6 labels only one component in each species. The molecular weight of proteins recognized by this antibody was 100kDa in maize extracts, 77 kDa in pollen tubes,180 kDa in mammalian cells (Vaughn and Harper 1998), and 78 kDa in *Allium* meiocytes (Schmit et al. 1994).

4.1.3 Centrin

Centrin is a 20 kDa, calcium binding phosphoprotein first identified as a contractile protein located in the striated flagellar roots associated with the basal bodies of green algae (Salisbury 1995). In fact, centrin is found in three basal body associated structures in the green alga, *Chlamydomonas*: the distal fibre that links adjacent basal bodies, the flagellar root, which attaches the basal body to the surface of the nucleus, and stellate fibres in the transition zone between the basal body and the flagella (Salisbury 1995). Members of the centrin family have since been reported in the centrioles, centrosomes and mitotic spindle poles in algae, higher plants, invertebrate and mammalian cells and the SPB in yeast cells (Salisbury 1995; Paoletti et al. 1996), although the report in plant cell mitotic spindle poles is controversial (see Del Vecchio et al. 1997; Vaughn and Harper 1998). Centrin resembles the well characterised calmodulin in size and in the fact that it has 2 calcium binding helix-loop-helix motifs called EF hands on each end of a central helix portion (Salisbury 1995).

In mammalian cultured cells, centrin is associated with MTOCs. Immunofluorescence staining reveals that centrin is tightly associated with centrioles all through the cell cycle but also has a diffuse cytoplasmic and nuclear localization; in early G1 the midbody is also slightly stained (Paoletti et al. 1996). In the centrioles of some cultured human cells, centrin was found in the lumen of the distal part of the centriole where the triplet structure changes to a doublet structure (Paoletti et al. 1996). This is strikingly similar to the reports that it is found in the luminal stellate fibres of the transition zone between the basal bodies and the flagellar axoneme (also a triplet to doublet transition) in both hepatics and green algae (Salisbury 1995; Vaughn and Harper 1998). Centrin is also found in the pericentriolar material in some mammalian cells that seems to respond to calcium concentrations (Baron et al. 1994; Salisbury 1995).

In lower plant cells, centrin is also associated with MTOCs in developing spermatogenous cells (Vaughn and Harper 1998). In *Chlamydomonas*, antibodies to centrin strongly label the spindle poles (Vaughn and Harper 1998).

In higher plants, the most conspicuous staining with centrin antibodies occurs in the developing cell plate during cytokinesis in both monocots and dicots (Del Vecchio et al. 1997; Vaughn and Harper 1998). In immunoelectron microscopy studies, this staining was associated with small vesicles and the cytoplasm, but not the phragmoplast microtubules (Del Vecchio et al. 1997). The authors suggest that this might indicate that centrin homologues are involved in some calcium mediated aspect of vesicle fusion in the formation of the cell plate. In immunofluorescence studies using antibodies to centrin, the nuclear envelope staining is variable, with slightly stronger staining in differentiating onion cells than in meristematic cells of onion and other angiosperms (Del Vecchio et al. 1997).

Centrin seems to be involved in a number of different cellular functions, some having only an indirect relation to microtubule organization. Its calcium dependent contractile features are exploited in the orientation of basal bodies in *Chlamydomonas* mentioned above (Harper et al. 1995). Another example of this is in the anaerobic protist, *Holomastigotoides*, which has centrin in a number of structures including flagellar bands that attach to and orient its 10,000 basal bodies (Lingle and Salisbury 1997). Centrin's conformational changes in response to calcium fluctuations may be instrumental in the coordinated beating of the thousands of flagella in this organism (Lingle and Salisbury 1997).

Another function related to the contractile features is flagellar severing. Centrin in the stellate fibers in the transition zone of the flagella of *Chlamydomonas* contracts in response to elevated Ca^{++} levels, severing the axonemal microtubules, resulting in the excision of the flagella in response to certain environmental or developmental situations (Sanders and Salisbury 1994; Paoletti et al. 1996). This severing capacity might also be utilized in MTOC duplication and separation. Studies using *Chlamydomonas* mutants reveal that centrin has an essential role in basal body separation (Salisbury 1995). In the yeast, *Saccharomyces cerevisiae*, the *CDC31* gene product is a member of the centrin family and is necessary for the initiation of the SPB duplication (Salisbury 1995; Paoletti et al. 1996). In pteridophyte blepharoplasts (which act as templates for centrioles at one stage of sperm development) centrin is present as the centrioles are being formed (Vaughn and Harper 1998).

In animal cells, the role of centrin in MTOCs is not as well characterized. Even though centrin is located in the distal lumen of vertebrate centrioles, it is still unknown what role or roles it plays in centrosome duplication or the coupling of this duplication with the end of mitosis in the cell cycle (Paoletti et al. 1996).

Centrin may also have microtubule nucleating functions. This possibility is based not only on its localization to centrosomal pericentriolar material or to other MTOCs but also on the report that incubating *Xenopus* egg extracts with antibodies to centrin interferes with microtubule nucleation. (reference cited in Vaughn and Harper 1998). However, this interference with nucleation by these antibodies could be due to steric hindrance and not to the blocking of a specific nucleating function of the centrin itself (Del Vecchio et al. 1997).

Because these three centrosomal constituents – γ-tubulin, the antigen recognized by 6C6 and centrin – act as microtubule nucleating markers in many organisms, we have used them to probe conifer cells, which represent an evolutionarily distinct group of higher plants.

4.2 Materials and methods

Preparation of root tip cells, and immunofluorescence microscopy on isolated whole cells was performed as described (2.2.3 and 2.2.4) except that a post-fixation in cold methanol (-20°C 10 minutes) was included in all preparations. Omitting the methanol step resulted in higher background and less specific staining, especially using 6C6. Exposure to cold methanol is necessary to uncover the epitope recognized by this antibody (M. Vantard, personal communication).

Affinity purified γ-41 and γ-9 antibodies raised against a peptide representing a conserved N-terminal sequence from *Aspergillus nidulans* (Walling et al, 1998) were gifts from T.H. MacRae. γ-9 recognized only centrosomes in mouse fibroblast cells and both stained a single band in cell free *Artemia* extracts with a molecular weight slightly lower than that of bands produced with an antibody to α-tubulin. The monoclonal IgM antibody 6C6 was a gift from A.M. Lambert's laboratory. The monoclonal anti-centrin antibody, 20H5 and the polyclonal antibody to fern γ-tubulin were gifts from J.D.I. Harper. The anti-centrin antibody has been well characterized and recognizes a 17-20 kDa protein in plant cells as well as a centrin in *Chlamydomonas* (Del Vecchio et al. 1997 and J.D.I. Harper, personal communication)

Conventional and confocal immuunofluorescence microscopy were performed as described (2.2.4).

4.3 Results

4.3.1 γ-tubulin

A γ-tubulin antibody, γ41, raised against a unique N-terminal sequence, stained specific structures in mitotic cells of both onion and pine. This antibody has been well characterized. It reacted with only centrosomes and not microtubules in mammalian cells and it recognized only one band corresponding to γ-tubulin on Western blots (personal communication T. H. MacRae). Figure 4.1a, a z-series of optical sections taken at 1 μm intervals through a metaphase/anaphase onion cell stained with the γ41 antibody, shows prominent staining at the broad poles and fine staining between the chromosomes. Figure 4.1b shows the results of a similar z series through a late anaphase-early telophase cell, with staining in the developing phragmoplast and peri-nuclear region. Interphase cells had diffuse cytoplasmic staining (not shown). Negative controls, with the secondary antibody only, showed no specific staining (Fig. 4.1c and 4.1d).

Staining with the γ41 antibody on pine root tip cells and observation with a confocal microscope revealed that this antibody reacted to specific structures. Figures 4.2a and 4.2b present results of a 1 μm z series through *Pinus radiata* cells. In late anaphase cells, poles were prominently stained. There was also some staining in the interzonal region (Fig. 4.2a). *Pinus radiata* metaphase cells had some staining in the area around the chromosomes (Fig 4.2b). This pattern with the γ41 antibody resembles that found with pine metaphase spindles stained with antibodies to α-tubulin (cf Fig 2.6a'), indicating that the γ-tubulin is associated with the spindle microtubules. Many interphase cells seemed to have weak staining at the cell cortex while others had staining around the nucleus (arrowhead in Fig 4.2a). Negative controls (even with enhanced contrast) showed only faint, non specific speckling over the whole cell (Figs. 4.2c, d).

Intense spindle staining patterns were seen in *Pinus contorta* cells treated with the γ41 antibody (Fig 4.3.a). Negative controls for this species were identical to those seen with *Pinus radiata* (not shown).

Attempts to use γ41 and other γ-tubulin antibodies with conventional fluorescence microscopy resulted in higher background, which made results more difficult to interpret. Even so, in a *Pinus radiata* metaphase cell double stained with γ9 and an antibody to β-tubulin, the γ-tubulin seems to coincide with the spindle stained with β-tubulin (Fig. 4.4.a,a'). In an interphase cell treated with the same antibodies, the γ9 seemed to stain the nucleus or nuclear envelope. This staining was quite distinct from the cortical microtubules stained with the β-tubulin antibody in the same cell (Fig. 4.4b, b'). Results using another γ-tubulin antibody, one raised against a fern sequence, were unexpected. This antibody, that had stained plant cells as described (Marc 1997), seemed to cross-react with *Pinus contorta* organelles distributed throughout the metaphase spindle (Fig 4.4c) and around the forming nuclear envelopes in telophase cells (Fig 4.4d). The results using γ41 observed with conventional fluorescence microscopy were not as clear as the results using the confocal microscope, although the phragmoplast was heavily stained (Fig 4.4e). Both γ41 and γ9 were raised against peptides found at the N-terminus of gamma tubulin. Attempts with antibodies raised against C-terminal peptides and to the fusion protein were unsuccessful using our plant material (not shown). Attempts using antibodies raised against γ-tubulin including γ41 and γ9 on methacrylate sections also failed to recognize microtubule (or any other) structures (data not shown). Preliminary results using LR White sections for immunoelectron microscopy were mixed. While some cortical microtubules in pine cells were occasionally stained using affinity purified γ9, microtubules in other structures such as spindles or phragmoplasts were not (data not shown).

4.3.2 6C6 localization in conifer cells

Results using the 6C6 antibody on conifer root cells indicate that this antibody reacted to specific, probably membrane, structures. In interphase cells, the antigen recognized by 6C6 was located at the nuclear envelope (Fig. 4.5c), in a similar distribution to that reported in angiosperm cells (Chevrier et al. 1992). In dividing conifer cells, the antibody stains the polar regions of the cells (Figs. 4.5a and 4.5b) and no kinetochore staining was observed. When methacrylate sections of pine root tips were stained using 6C6, some staining of the nuclear envelope was visible (not shown). Negative controls using secondary antibodies only or normal mouse serum had no specific staining (not shown).

4.3.3 Centrin localization in conifer cells

Figure 4.6 illustrates the distribution of a centrin homologue recognized by the antibody, 20H5 in *Pinus contorta* root tip cells and observed with conventional immunofluorescence. In a late anaphase cell, there is labelling between the separating chromosomes as well as some other cytoplasmic staining (Fig. 4.6a). Figures 4.6b, 4.6c and 4.6d depict examples of the most consistent labelling pattern with this antibody on conifer cells – a strong specific labelling of the developing cell plate. Note also that there was a consistent faint cytoplasmic stain, probably the result of a pool of cytoplasmic centrin. There was no noticeable staining of the nuclear envelope.

Figure 4.1. Root tip cells of *Allium cepa* stained with γ41, an affinity purified antibody to a conserved sequence of *Aspergillus nidulans* γ-tubulin (**a,b**) or negative controls (**c,d**), **a',b',c',d'** corresponding nuclei stained with propidium iodide. CLSM images. All micrographs are at the same magnification, bar = 10 μm.

a - A z-series of optical sections taken at 1 μm intervals through a cell in late metaphase/ early anaphase. One pole is marked with an arrowhead in the uppermost section.

b - A z-series of optical sections taken at 1 μm intervals through a cell in late anaphase / early telophase. One pole is marked with an arrowhead in the uppermost section.

c - A 1μm section through a group of cells in interphase treated with secondary antibody only.

d - A 1μm section through a cell in anaphase treated with secondary antibody only.

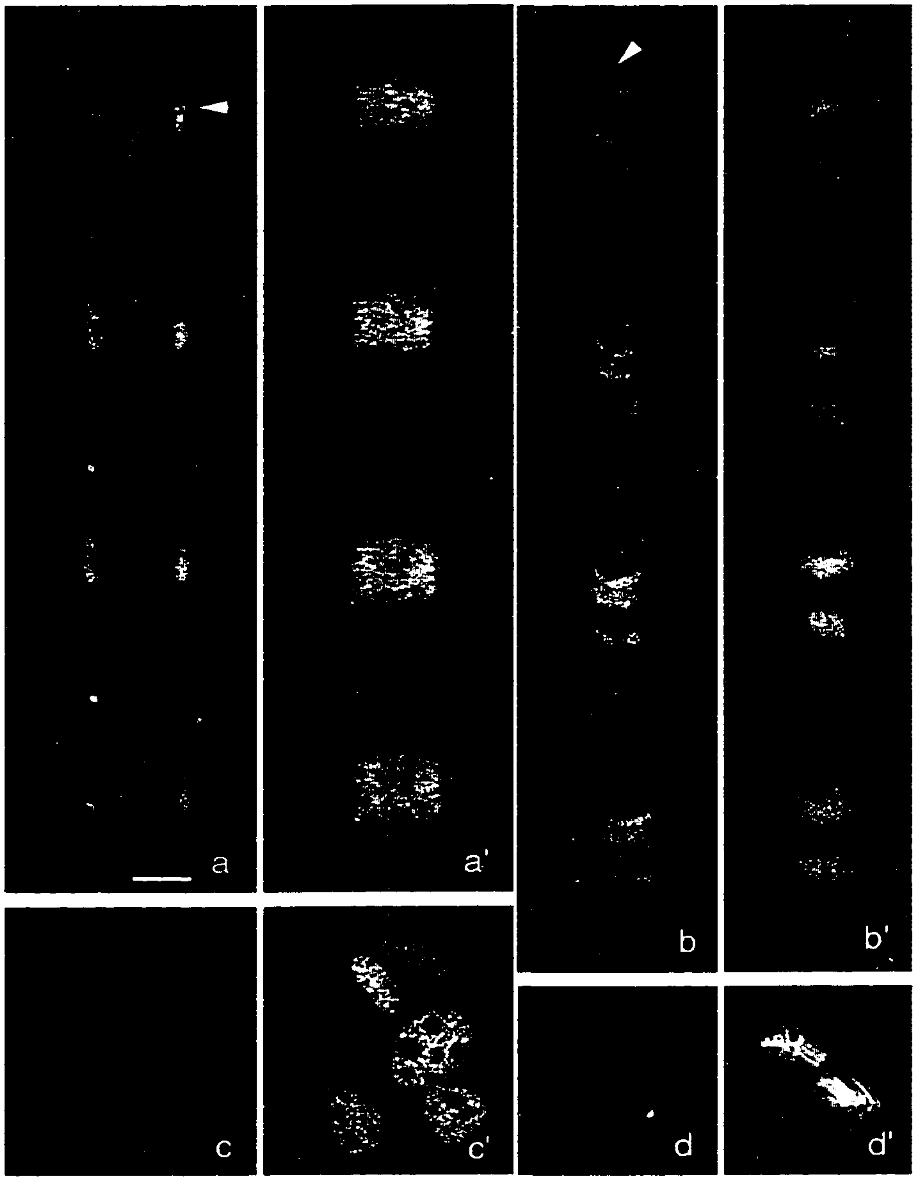

a
a'
b
b'
c
c'
d
d'

Figure 4.2. Root tip cells of *Pinus radiata* stained with γ41, an affinity purified antibody to a conserved sequence of *Aspergillus nidulans* γ-tubulin (**a,b**) or negative controls (**c,d**), **a',b',d'** corresponding nuclei stained with propidium iodide. CLSM images. All micrographs are at the same magnification, bar = 10 μm.

a - A z-series of optical sections taken at 1 μm intervals through two cells: a cell in late anaphase / early telophase and a cell in interphase. An interphase cell with nuclear envelope labelling is marked with an arrowhead in the uppermost section.

b -A z-series of optical sections taken at 1 μm intervals through a cell in metaphase.

c - A 1μm section through a cell in telophase treated with secondary antibody only.

d - A 1μm section through a cell in metaphase treated with secondary antibody only.

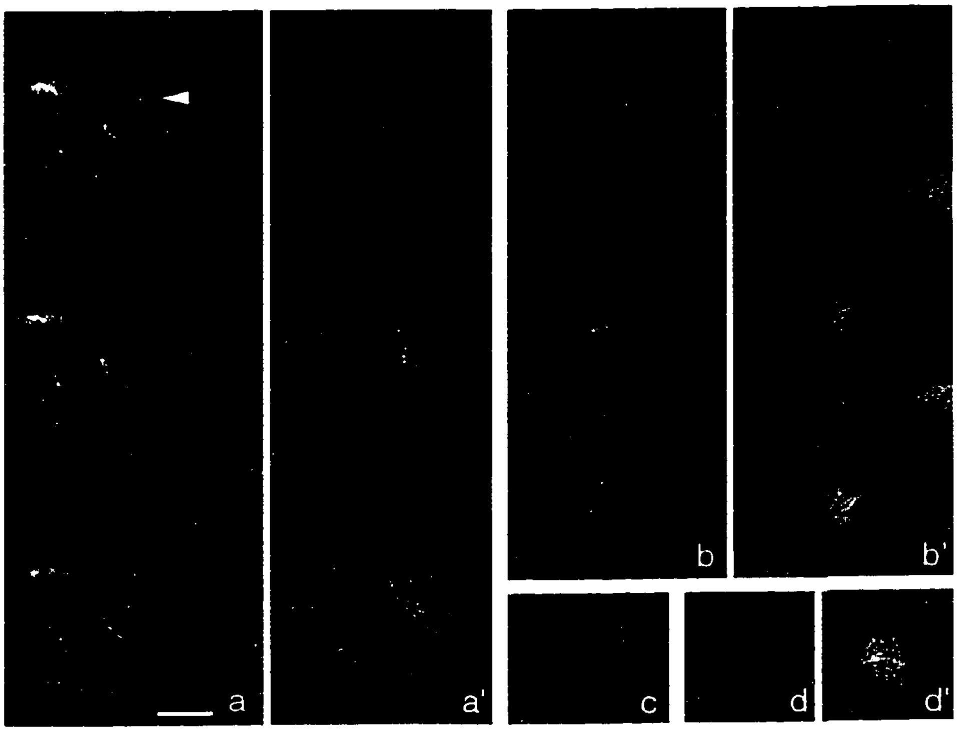

a
a'
b
b'
c
d
d'

Figure 4.3. A root tip cell of *Pinus contorta* stained with γ41, an affinity purified antibody to a conserved sequence of *Aspergillus nidulans* γ-tubulin (**a**), **a'** corresponding nucleus stained with propidium iodide. CLSM images. Bar = 10 μm.

a -A z-series of optical sections taken at 1 μm intervals through a cell in metaphase.

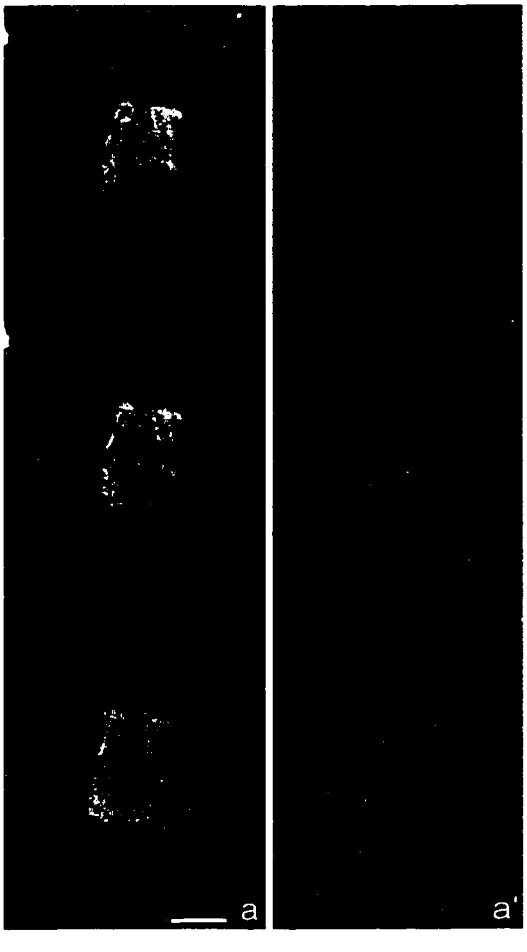

a
a'

Figure 4.4. Root tip cells of *Pinus sp.* stained with γ9, an affinity purified antibody to a conserved sequence of *Aspergillus nidulans* γ-tubulin **(a,b)** or to a monoclonal antibody to β-tubulin **(a',b')** or to an antibody to a conserved sequence of plant γ-tubulin **(c,d)**, or to γ41 an affinity purified antibody to a conserved sequence of *Aspergillus nidulans* γ-tubulin (e) **a'',b'',c',d'** corresponding nuclei or chromosomes stained with DAPI. All micrographs are at the same magnification, bar = 10 μm.

a - A *Pinus radiata* cell in metaphase stained with γ9, arrowhead indicates a kinetochore fibre.

a' -The same cell as in **a**, double stained with an antibody to β-tubulin, arrowhead indicates the same kinetochore fibre indicated in **a**.

b - A *Pinus radiata* cell in interphase stained with γ9.

b' -The same cell as in **b**, double stained with an antibody to β-tubulin, arrowhead indicates cortical microtubules .

c - A *Pinus contorta* cell in metaphase stained with an antibody to a conserved sequence of plant γ-tubulin.

d - A *Pinus contorta* cell in telophase stained with an antibody to a conserved sequence of plant γ-tubulin.

e - Two *Pinus contorta* cells stained with γ41: one cell in telophase, one in interphase.

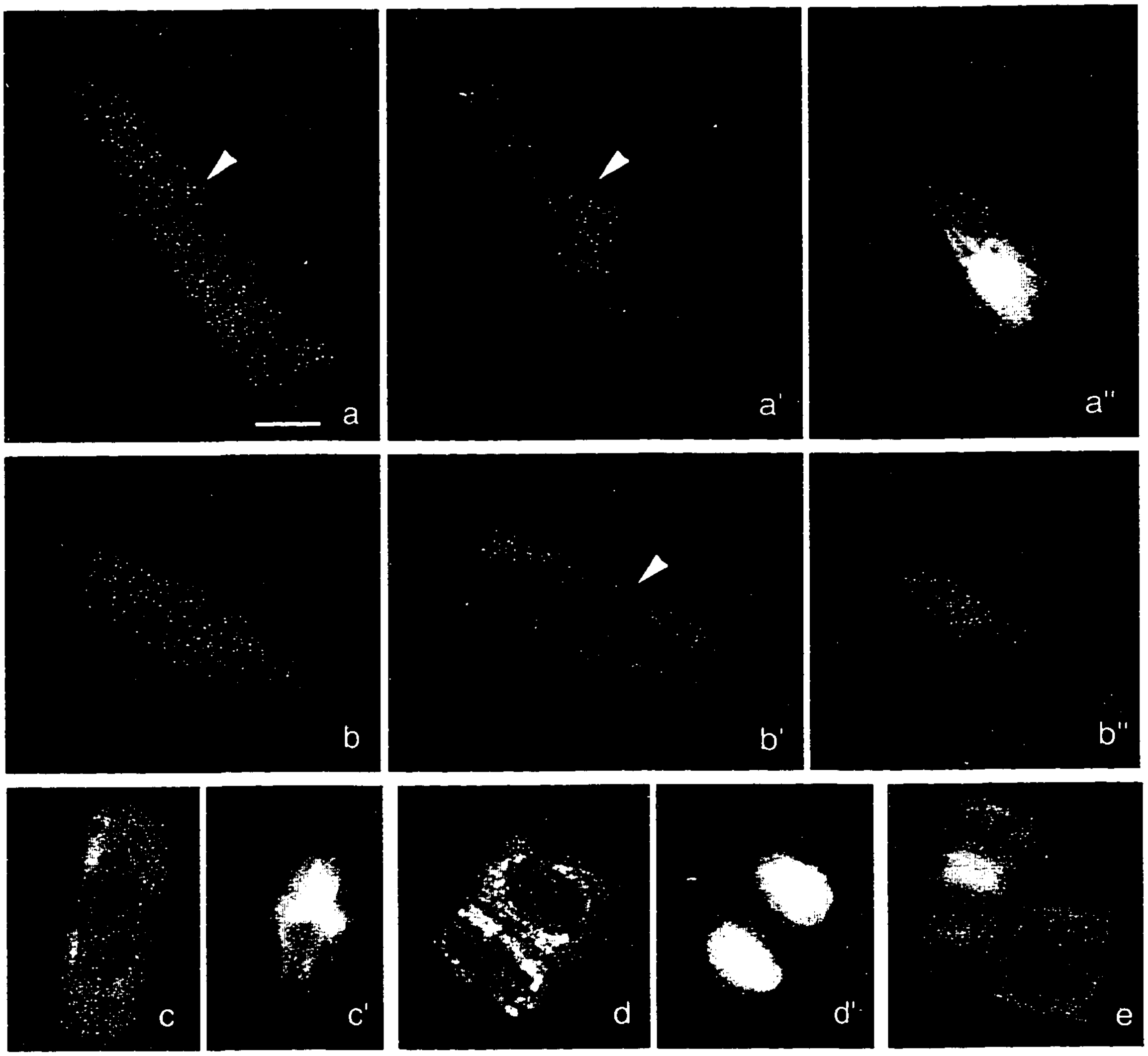

a
a'
a''
b
b'
b''
c
c'
d
d'
e

Figure 4.5. Root tip cells of *Pinus sp.* stained with 6C6, a monoclonal antibody raised against a component of calf thymus centrosomes **a',b',c'** corresponding nuclei stained with DAPI. All micrographs are at the same magnification, bar = 10 μm.

a - A cell in metaphase stained with 6C6. Note strong labelling at the poles.

b - A cell in anaphase stained with 6C6. Note strong labelling at the poles.

c - A group of cells in interphase stained with 6C6. Arrowheads indicate staining on the nuclear envelope.

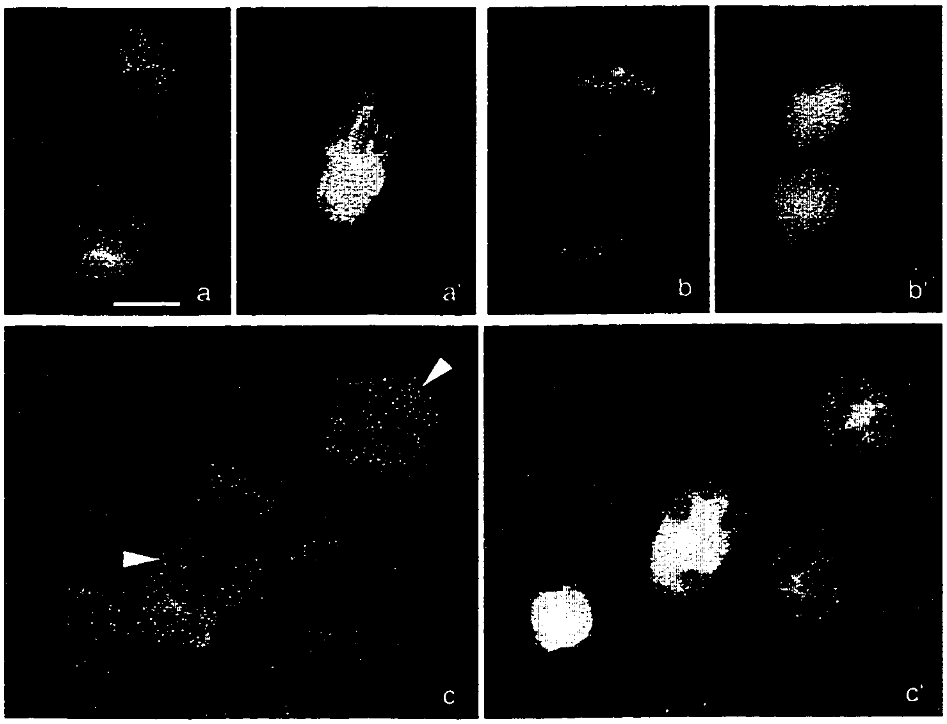

a
a'
b
b'
c
c'

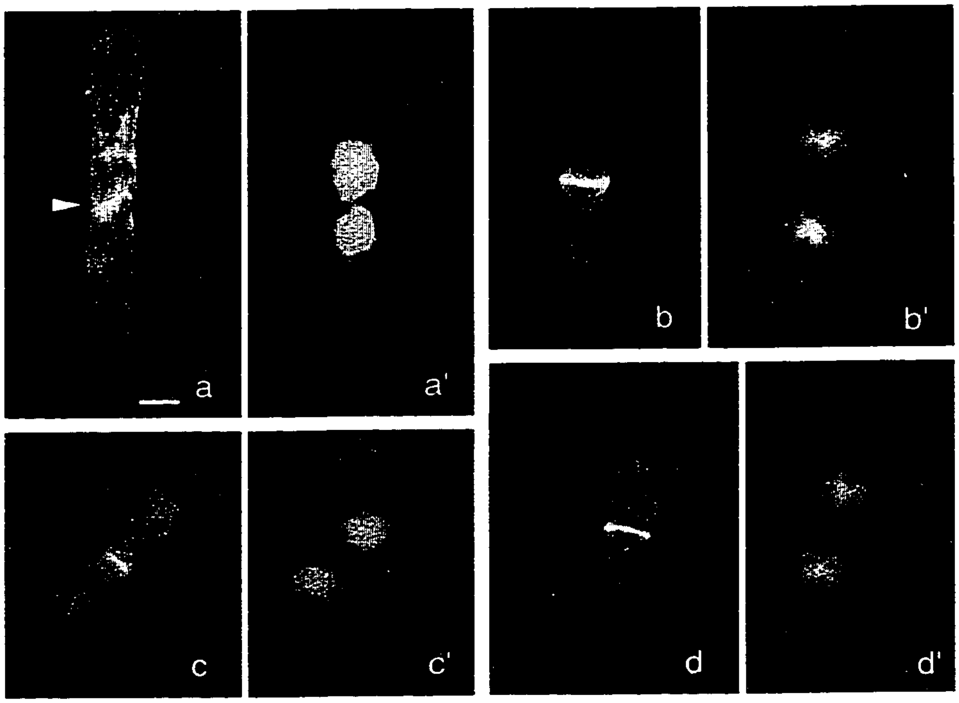
Figure 4.6. Root tip cells of *Pinus contorta* stained with 20H5 a monoclonal anti-centrin antibody (**a-d**). **a',b',c',d'** corresponding nuclei stained with DAPI. All micrographs are at the same magnification, bar = 10 μm.

a - A cell in anaphase stained with 20H5, arrowhead indicates region between separating chromosomes.

b - A cell in early telophase stained with 20H5, showing strong labelling of the cell plate.

c - A cell in early telophase stained with 20H5, showing strong labelling of the cell plate.

d - A cell in early telophase stained with 20H5, showing strong labelling of the cell plate.


a
a'
b
b'
c
c'
d
d'

4.4 Discussion

Attempts to characterize higher plant MTOCs or nucleating sites by using antibodies as probes have not been as straightforward as might have been anticipated, but still this method has been called the most promising method in elucidating the nature of amorphous higher plant MTOCs (Del Vecchio et al. 1997).

Our results with γ-41 on *Allium* root cells are similar to those reported earlier (Liu et al. 1993; Marc 1997) and confirm that this antibody recognizes plant γ-tubulin. There is a close resemblance between the staining of the polar regions of the early anaphase/late metaphase cells in this study and the pattern of staining of anaphase *Allium* root cells in the earlier reports. The staining of telophase cells was also similar to earlier reports of γ-tubulin antibody staining of the phragmoplast microtubules. Both laboratories also presented data indicating some perinuclear staining at telophase, although overall staining at telophase was more abundant in our results than those reported by either group (Liu et al. 1993; Marc 1997).

The fact that the antibody to γ-tubulin recognizes microtubule arrays in conifer species confirms and extends the results reported in angiosperms. This indicates that in both groups of higher plants, the distribution of γ-tubulin is much more extensively associated with microtubules than it is in animal cells. This does not preclude the possibility that γ-tubulin is involved with microtubule nucleating. In plant cells, γ-tubulin very well might be present in nucleating sites, as staining of the nuclear envelope and the cell cortex indicates, but the fact that staining is also present in microtubule structures complicates the interpretation of staining results, and makes the identification of plant nucleating sites by the localization of γ-tubulin problematic.

Staining with 6C6 revealed important similarities and also differences between the results with conifer cells in this study and results with angiosperm cells reported earlier (Chevrier et al. 1992; Schmit et al. 1994). In all of the plant species reported so far, the 6C6 antibody has specifically stained the nuclear surface when the latter was intact (in interphase, early prophase and late telophase). This labelling seems to correspond with the microtubule nucleating site on the envelope (Lambert 1993; Stoppin et al. 1996; Vaughn and Harper 1998). However, after the nuclear envelope breaks down, the 6C6 antigen seems to move to the kinetochores during mitotis in angiosperm cells (Schmit et al. 1994). When the nuclear envelope is broken down in conifer cells, this antigen seems to remain with the endomembranes as indicated by the concentrated label near the poles. The indication that this antigen does not migrate in conifer cells may be the result of a tighter association with the conifer nuclear membrane or a reduced ability of the conifer kinetochore to transport this protein (Schmit et al. 1994).

The labelling with the centrin antibody in the conifer cells in this study was very similar to the labelling reported in angiosperm cells (Del Vecchio et al. 1997). In both onion cells (Del Vecchio et al. 1997) and pine cells, there was some signal between the chromosomes in anaphase. In all cells studied, including *Pinus contorta* in this study, centrin homologues are localized to the developing cell plate. There was little or no label detected on the nuclear envelope in the pine cells; however, a faint signal would be difficult to detect without viewing with a confocal microscope.

Centrin is associated with MTOCs in other organisms, but there is no real evidence that it is labelling a similar site in conifer cells, or indeed in other higher plant cells. In spite of its association with structural MTOCs, there is little evidence that centrin is directly involved in either microtubule nucleation or microtubule organization in animal or lower plant cells. Rather centrin is probably present in the MTOC in conjunction with its well defined role in the processes of MTOC duplication and separation. This, however, is only necessary in those organisms that have centrioles that have to be severed and moved. It probably does not need to function in the same way in plant MTOCs where the nucleating role is associated with a much more amorphous, probably membrane associated structure. The centrin in cell plates is not localised to putative MTOCs in the phragmoplast (unpublished Vaughn, in Del Vecchio et al. 1997), but may be involved in the calcium-mediated fusion of vesicle membranes. In support of this, immunoelectronmicroscopy cytochemical localization of centrin indicates that it is associated with vesicles in the phragmoplast. The cell plate formation is sensitive to calcium disruption, therefore centrin may be involved in calcium sequestering or other calcium modulating activities (Del Vecchio et al. 1997). Centrin may not be involved in microtubule nucleation or microtubule organization in higher plants. Instead, its calcium binding ability may be utilized in calcium signaling in a myriad of different processes (Del Vecchio et al. 1997; Vaughn and Harper 1998). This multi- functional aspect of centrin is supported by reports that in animal cells over 90% of the centrin is not localized to centrosomes (Paoletti et al. 1996; Vaughn and Harper 1998), and the suggestion that it may be involved in Ca^{++} signaling pathways (Del Vecchio et al. 1997).

In all three parts of this study, we have attempted to characterize microtubule nucleating sites in conifer cells by probing isolated root cells with antibodies developed against conserved centrosomal proteins. In spite of the fact that the results did not conclusively identify particular nucleating sites, a number of interesting observations have been made. The distribution of the antigens corresponding to all of the antibodies used in this study were similar to the distribution reported in angiosperms. This suggests, not surprisingly, that these components are utilized in a similar way in both groups of higher plants, even though there are superficial differences like spindle shape (Wang et al. 1991) between the groups. However, the distribution of γ-tubulin and centrin homologues in plant cells, including conifer cells, indicates that their roles may be more diverse and complex than was originally thought (Vaughn and Harper 1998). This illustrates the sometimes unexpected benefits of this method of research. Localization of these and other putative MTOC components in higher plant cells will not only contribute to our understanding of microtubule organization in plants but also clarify the roles that these components play in microtubule organization generally.

4.5 References

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Chapter 5. MPM-2 Staining in *Marchantia* cells

5.1 Introduction

One aspect of MTOCs not emphasized in the previous chapter is that their microtubule nucleating actions are coordinated with other cellular processes. One of the major coordinating points in the cell cycle of eukaryotes is the entry into mitosis. During mitosis the cellular changes are profound: chromatin condenses into chromosomes, the nuclear envelope breaks down, and throughout the process, the cytoskeleton completely reorganizes. Entry into mitosis is governed by a kinase complex called the M-phase promoting factor (MPF). The activation of MPF triggers a phosphorylation cascade that directly or indirectly induces the changes associated with mitosis. Homologues of the components of MPF (a mitosis specific cyclin and $p34^{cdc2}$, a cyclin dependent protein kinase) are so highly conserved among eukaryotes that the human gene for a $p34^{cdc2}$ homologue can substitute for its yeast counterpart (reviewed in Doonan 1991).

In spite of the fact that many of the components of the cell cycle regulation in animal and yeast cells have been identified, less information exists about cell cycle regulation in plants. However some key components have been found. These include plant structural and functional homologues of $p34^{cdc2}$, cyclin, and a few other cell cycle regulatory components (John et al. 1990; Mineyuki et al. 1991; Staiger and Doonan 1993). Recently, an inhibitor of $p34^{cdc2}$ kinase also has been identified in *Arabidopsis* (Wang et al. 1997).

Our understanding of plant cell cycle regulation is assisted by information on the analogous process in animal and yeast cells (Staiger and Doonan 1993). This is possible because similarities do exist between plant and other eukaryotic cells. These include: (1) the existence of homologous kinases, which are key players in this regulation; (2)

evidence of a similar phosphorylation cascade triggering mitosis onset (Staiger and Doonan 1993); and, (3) recognition of a number of the resulting phosphorylated proteins by a monoclonal antibody, MPM-2, raised against mitotic mammalian cells. In animal and yeast cells, this antibody recognizes MTOCs in interphase and a number of other structures as these cells enter mitosis. In all cells, including plant cells, staining by MPM-2 dramatically increases as cells enter mitosis. In higher plant cells, MPM-2 recognizes a number of components during mitosis. Some of these are connected with the cytoskeleton and will be discussed below.

In this chapter, the presence and distribution of antigens recognized by MPM-2 in dividing cells of *Marchantia polymorpha* was investigated. This lower plant, like other bryophytes, has a recognizable MTOC at one stage of the cell cycle (Fowke and Pickett-Heaps 1978; Steer 1984). This aspect of the investigation of MTOCs in plant cells was undertaken to see if MPM-2, which recognizes plant and fungal MTOCs, would react with the structurally unique polar organizer, that appears to nucleate microtubules during *Marchantia* prophase. In this study, the distribution of this phosphoepitope during the cell cycle was documented and compared to higher plant cells including those of the conifer, *Pinus radiata.*

5.1.1 MPM-2

MPM-2 is one of a number of antibodies raised 15 years ago against proteins in mitotic Hela cells (Davies et al. 1983). It recognises a number of phosphorylated proteins that share a similar epitope in animal, fungal and plant cells and appear in a cell cycle specific manner (Traas et al. 1992; Wolniuk and Larsen 1992; Rose et al. 1993; Binarova et al. 1993). In all phyla studied so far, this reactivity increases during G2, reaches a peak during metaphase, and abruptly declines during anaphase (Davies et al. 1983; Vandre et al. 1984; Traas et al. 1992; Rose et al. 1993). We know that the MPM-2 antigen contains a phosphate group because the epitope can be destroyed by phosphatases (Davies et al.

1983; Traas et al. 1992). We also know that the phosphoepitopes of at least some of these antigens are important for entry into mitosis and microtubule nucleation. Treatment of mammalian interphase cells or centrosomes with MPM-2 blocks entry into mitosis or nucleation of microtubules respectively (Rose et al. 1993; Kuang and Ashorn 1993).

The phosphorylation of some of the MPM-2 epitopes is most likely controlled through the action of the ubiquitous $p34^{cdc2}$ kinase (Verde et al. 1990), but this kinase probably does not act directly on at least most of the MPM-2 substrates, but rather on other kinases that in turn phosphorylate the MPM-2 proteins (Kuang and Ashorn 1993). However, the control of the activation of the $p34^{cdc2}$ kinase is also the result of the activities of phosphorylated, MPM-2 recognized proteins. Both Cdc25 and Wee1, which activate and inhibit the activity of $p34^{cdc2}$ kinase, respectively, are recognized by MPM-2 in their phosphorylated, activated states (Kumagai and Dunphy 1996; Shen et al. 1998). It was originally believed that a single kinase created the MPM-2 epitope in a number of key M-phase regulatory proteins; however in spite of considerable effort, a single MPM-2 kinase has not been identified. A number of kinases have been implicated including MEK, MAPK, and $p34^{cdc2}$ kinase itself (Kuang and Ashorn 1993; Renzi et al. 1997; Ding et al. 1997).

The MPM-2 epitope has recently been defined by analysing known MPM-2 phosphoepitopes, and artificially creating and analysing variations of these (Ding et al. 1997). The MPM-2 antibody recognizes a very specific epitope – a phosphorylated amino acid (threonine or serine) flanked by an aromatic amino acid residue that must be 2 or 3 residues to the N-terminal side and an aromatic or positively charged amino acid to the C-terminal side (Ding et al. 1997). This definition, and the artificial peptides created in this research, should be useful in finally identifying kinases responsible for the MPM-2 epitope.

Although some of the kinases responsible for the MPM-2 phosphoepitopes are not known, some of the proteins that are recognized by MPM-2 have been identified. These include proteins directly involved with the cytoskeleton such as microtubule associated proteins (MAPs) (Smirnova et al. 1995), proteins that associate with chromosomes (Taagepera et al. 1993), and others important in cell cycle regulation: Cdc25, Myt1, Wee1, Plk1, and Cdc27. (Kumagai and Dunphy 1996; Shen et al. 1998). γ- tubulin is not a major MPM-2 epitope nor is it necessary to bind MPM -2 epitopes to the spindle pole body (SPB) in yeasts, even though both MPM-2 reactive phosphoepitopes and γ-tubulin are required for microtubule nucleation and both are located at SPBs (Oakley 1995).

A large number of mammalian proteins are recognized by this monoclonal antibody; they range in size from 40 to 200kDa (Davies et al. 1983). Most remain unidentified in the 15 years since the antibody was produced. In spite of the similarity in the timing of the appearance of these epitopes, the structures that show MPM-2 reactivity and the specific phosphoproteins that contain this epitope differ between phyla. For example, the major chromosome protein that is recognized by this antibody in animal cells is topoisomerase II (Taagepera et al. 1993), but this has not been identified as such on immunoblots using this antibody on higher plant material (Traas et al. 1992).

Vandre et al. (1984) found that mammalian interphase centrosomes, and probably the centriole portion of the centrosome only, reacted with MPM-2. Interphase nuclei also contained this phosphoepitope. When the nuclear envelope broke down, the reactivity spread through the cytoplasm. In mammalian cells, much of the cytoplasmic reactivity can be extracted and is therefore soluble, but some remains on microtubule structures and MTOCs such as the spindle poles, kinetochores and later the midbody (Vandre et al. 1984). Staining of these structures decreases precipitously at the onset of anaphase (Vandre and Borisy 1989). Recently some of the kinases responsible for the kinetochore MPM-2 epitopes of PtK_1 cells were analysed (Renzi et al. 1997). By careful analysis of a variety of kinase inhibitors these authors determined that multiple kinases, soluble or kinetochore bound, can create the MPM-2 phosphoepitope. By treating the kinetochores with phosphatases, and then attempting to rephosphorylate them with known purified kinases, they were also able to determine that $p34^{cdc2}$-cyclin B and MAP kinase do not create the MPM-2 epitope at kinetochores, while the *Aspergillus* kinase, NIMA, can create this epitope on these mammalian kinetochores (Renzi et al. 1997). This indicates that a mammalian NIMA homologue operates at the kinetochores of mammals (Renzi et al. 1997). These MPM-2 kinetochore phosphoepitopes were also temporally regulated with other phosphoepitopes. MPM-2 reactivity in the kinetochores of PtK1 and mouse P388D1 cultured cells were found primarily in M phase, and were distinct from other phosphotyrosine proteins (not recognized by MPM-2) found in prophase and prometaphase only (Taagepera et al. 1995).

In human medical research, MPM-2 has been used in applications that take advantage of its role as an indicator of cell cycle related phosphorylation. MPM-2 may be useful as a proliferation biomarker in assessing cervical cone biopsy specimens (Hu et al. 1997). And recently, MPM-2 was shown to react to neurofibrillary tangles, neuritic processes, and neurons in brains of patients with Alzheimer's disease, but not in normal brain tissue. This indicates that the widespread hyperphosphorylation of proteins in this disease may result from the activation of mitotic phosphorylation mechanisms that do not normally operate in neurons (Vincent et al. 1996).

In yeast, MPM-2 reacts with the spindle pole body, the yeast MTOC; this reactivity appears during G2 and continues through mitosis. The appearance of the MPM-2 epitope precedes spindle formation (Engle et al. 1988).

5.1.2 MPM-2 staining in plant cells

The reactivity of MPM-2 in plants has not been as convincingly tied to MTOC sites although the temporal pattern of reactivity has been exactly the same as for mammalian cells (lowest in G1 and S phase cells, rising in G2 and highest during prophase and metaphase, dropping in anaphase (Traas et al. 1992). The nuclear surface, which is believed by many to be the main MTOC in higher plant cells (Lambert 1993), shows no MPM-2 immunoreactivity in any study so far (Traas et al. 1992; Binarova et al.1993, 1994), although this may be due to detergent membrane permeabilizing procedures that are routine for immunofluorescence studies (Binarova et al. 1993; Hoffman et al. 1994). One study that did show nuclear envelope immunoreactivity occurred using green algae and indicated that the antibody is reacting to phosphorylated karyoskeletal proteins, probably lamins, and therefore presumably not to microtubule nucleating proteins (Harper et al. 1990). Incidently, this recognition of a possible intermediate filament related protein by MPM-2 was also seen by Traas et al. (1992) when one protein on a 2D blot was recognized by both MPM-2 and a general intermediate filament antibody. Another possible MTOC in higher plants, the pointed spindle poles of gymnosperms, also did not show MPM-2 reactivity (Binarova et al. 1994).

One plant MTOC that does contain the MPM-2 phosphoepitope is the blepharaplast, a centriole-like MTOC found in pteridophyte spermatogenous cells (Hoffman et al. 1994, Vaughn and Harper 1998). This structure was labelled by MPM-2 in an immunogold electron microscopy experiment testing a number of antibodies to putative MTOC components (Hoffman et al. 1994). The authors were fairly confident that the MPM-2 antigen was located in the material that was nucleating microtubules. No other structure was recognized by MPM-2, except for the interphase nuclei and the mitotic cytoplasm in this lower plant.

Mitotic structures with MPM-2 reactivity in higher plants are kinetochores and phragmoplasts, although these structures have not been immunolabelled in all higher plant studies (Traas et al. 1992; Binarova et al.1993, 1994). While neither kinetochores nor phragmoplasts are classic minus-end MTOCs there is some evidence that these structures can nucleate microtubules (see Seagull 1989; Lambert 1993).

Two recent studies of MPM-2 labelling in higher plants extend and challenge some of the earlier reports. In microspores and pollen in *Brassica napus* cv. Topas, MPM-2 labelled the nucleoplasm in both the G_1 arrested vegetative nucleus and the dividing generative nucleus (Hause et al. 1995). This unexpected labelling of the non-dividing vegetative nuclei as well as the dividing generative nuclei shows that the phosphoepitope recognized by MPM-2 is present in high quantities in some G_1 cells, unlike the results reported in other plant and animal cells. This study also provided evidence that the change in developmental fate between gametophytic and sporophytic development of pollen or microspores was not correlated with changes in MPM-2 detectable phosphorylation (Hause et al. 1995). No MPM-2 labelling of microtubules was observed in these pollen grains or microspores (Hause et al. 1995).

The only report of microtubule labelling by MPM-2 was one that occurred in mitotic endosperm cells (Smirnova et al. 1995). In these unusual cells, the epitope recognized by MPM-2 was present in interphase in cytoplasmic granules and the nucleus; in prophase, MPM-2 immunoreactivity appeared in the nucleus, at the nuclear envelope and on microtubules, and there was an increased immunoreactivity in the cytoplasm; later in mitosis, MPM-2 staining occurred in the cytoplasm, mitotic spindle microtubules, phragmoplast and the newly formed cell plate; after mitosis, the cell plate and the cytoplasmic granules were the only structures that stained with this antibody (Smirnova et al. 1995). This apparent staining of microtubules could actually be the staining of a MAP that is phosphorylated during mitosis in these specialized cells. In fact, the cells used in this study were fixed in a manner similar to methods routinely used for animal cells, in cold methanol only, rather than the formaldehyde-first method used in plant cells (Smirnova et al. 1995). The authors noted that this method was particularly appropriate for the fixation of MAPs. The proteins recognized by MPM-2 in blots had molecular masses similar to those of plant MAPs. The authors subscribe to the opinion that plant microtubules have special properties that allow them to self-assemble without a separate MTOC (discussed in Balczon 1996). They also consider the reactivity of MPM-2 (which is localized to MTOCs in animals) along microtubules to be analogous to the similar association of γ-tubulin and other animal MTOCs with plant microtubules and to indicate differences in microtubule nucleation/organization between higher plant and animal cells (Smirnova et al. 1995). It must be emphasized that this is the only report of MPM-2 staining of microtubules in any cells, plant or animal. Further studies using the methods used in this study on a range of plant material will need to be undertaken to see if these results can be duplicated in non-endosperm plant material.

The nucleolus has been reported to be immunoreactive with MPM-2 in two studies using plant material (Binarova et al. 1993, 1994), but not in others (Traas et al. 1992). There have not been reports of this staining pattern in mammalian cells. In this regard, it is interesting that one isoform of topoisomerase II has been localized to the nucleolus in mammalian cells (Zini et al. 1992). This highly conserved enzyme is involved in many aspects of DNA function, such as the initiation of replication, replication itself and the condensation of chromatin into chromosomes, through its ability to alter DNA topology (Taagepera et al. 1993). Another isoform of this enzyme contains the MPM-2 epitope in mammalian cells (Taagepera et al. 1993).

In summary, even though there are waves of phosphoepitopes during the onset of mitosis in higher plant cells, there is conflicting evidence that phosphoproteins recognized by MPM-2 are associated with plant nucleating structures. Because only one example of MPM-2 staining of gymnosperms has been reported (Binarova et al. 1994), in this study we looked at the distribution of staining in cells of another conifer, *Pinus radiata*. In lower plants, one structural MTOC is stained with MPM-2 (reviewed in Vaughn and Harper 1998). In this study we probe a putative MTOC in another lower plant, *Marchantia*, using the antibody MPM-2.

5.1.3 *Marchantia polymorpha*

Marchantia polymorpha is a liverwort that is a common weed in greenhouses. It reproduces vegetatively by means of gemmae that form in gemmae cups on the upper surface of the gametophytic thallus, or by spores produced by the sporophyte embedded in the archegonium. Mitosis in this bryophyte has many similarities with that in vascular plants: open mitotic spindles, phragmoplasts, and preprophase bands (albeit 'poorly developed', Fowke and Pickett-Heaps 1978). It also has unique features found only in hepatics: nuclear shaping and extra nuclear polar bodies that appear during prophase (Fowke and Pickett-Heaps 1978; Steer 1984; Ducket 1986). Even though bryophytes are no longer believed to be a link in a direct line of descent between algae and vascular land plants (Ducket 1986), they do represent a primitive and unique plant system. The hepatic prophase nucleus becomes spindle shaped and is associated with microtubules that run lengthwise just above the nuclear surface and curve into the polar bodies that lie at either end (Steer 1984). These polar organizers are acentriolar, they are associated with endoplasmic reticulum and vesicles, and they have an amorphous, electron dense matrix (Brown and Lemmon 1990). They differ from those MTOCs that have centrioles in that they are transient and they disappear during the transition from prophase to metaphase, showing no evidence of division or migration (Brown and Lemmon 1990). These polar organizers are one of the few examples of identifiable structures besides nuclear envelopes which seem to be MTOCs in plant cells. This unique structure may represent an example of a step in the evolution of the diffuse, membrane associated higher plant MTOC. Another example is the MTOC feature of the plastid envelope in those lower plants having mono-plastidic cells (Brown and Lemmon 1993).

There are two possible sources for mitotic material in *Marchantia*. One is the notch area of the growing gemmling (Fowke and Pickett-Heaps 1978), the other is the meristematic region at the top of the stalk of the archegoniophore or antheridiophore (van den Heuvel and Harlow 1993).We have chosen the former, because of the ease of obtaining material on an ongoing basis. In spite of the fact that *Marchantia* is extremely easy to grow, there are some problems associated with the study of this plant. Bryophyte material has been found to be difficult both to infiltrate with resins for EM, and to permeabilize for immunofluoresence (Ducket 1986). In addition, *Marchantia* has relatively small cells and high levels of autofluorescence.

5.2 Material and Methods

5.2.1 Plant Material

Marchantia polymorpha:

The notch regions of *M. polymorpha* gemmae (grown on soil under 16 hour days for 8 days) were excised into fixative and prepared for methacrylate sections or EM as described in Chapter 2 (2.2.2, 2.2.5). Attempts to use meristematic material at the top of the stalks of the archegoniophore and antheridiophore were abandoned because no dividing cells were found in these structures when they were found as weeds in commercial plants, and attempts to induce growth of these structures were unsuccessful.

Pinus radiata:

Pinus radiata was grown and prepared for methacrylate sections or isolated whole cells as described (2.2.2, 2.2.3).

5.2.2 Immunofluorescence staining of methacrylate sections and isolated cells

This was performed as described in Chapter 2 (2.2.4) except that the primary antibody was a 1/300 dilution of MPM-2. For DNA staining of *Marchantia* sections, a much higher concentration of DAPI was needed than was reported above for other plant material. In the results reported here, the stock solution (1mg/ml) was applied directly onto the wells containing the sections and approximately 15-20 µls of PBS, giving a final concentration of DAPI of at least 0.25 mg/ml. Sections were incubated in the dark for 5 minutes and then washed as described above.

5.2.3 Preparation of Araldite sections for EM

One mm^3 portions of tissue containing the apical notch region of 8 day old gemmlings were excised and fixed sequentially in 1% and 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8) for 1 hour and 2 hours respectively at room temperature. After 4 washes in the phosphate buffer, samples were postfixed in 1% osmium tetraoxide in phosphate buffer overnight (this and subsequent steps were performed at 0°C), then washed 2 times in ddH_2O. Tissue was dehydrated to 100% ethanol by 10% increments (30 minutes each) followed by 3 changes in dehydrated 100% ethanol. Propylene oxide was gradually added over 6 hours until it represented 3/4 of the total volume, then three changes of propylene oxide were made of ½ hour each. Tissue was left in propylene oxide overnight. Material was brought to room temperature for the rest of the procedure and propylene oxide was removed, leaving only enough to cover the tissue. Vials containing the tissue were put in an apparatus that slowly rotated. A mixture of 1:1, Araldite resin: propylene oxide was added by drops over 4 hours. The resulting resin mixture was removed, replaced by a 2:1 Araldite resin: propylene oxide mixture and left overnight on the rotator at room temperature. At this point, vials were removed from the rotator, uncovered except for perforated foil caps and left in a fume hood for 6 hours to allow the propylene oxide to evaporate. Samples were transferred to fresh Araldite resin and then to silicon embedding
moulds, and left 24 hours before polymerizing at 60°C for 48 hours.

Silver sections were cut with a diamond knife, stained for 15 minutes with uranyl acetate in 70% ethanol and 5 minutes in Reynold's lead citrate and examined using a 420 or CM10 model Phillips electron microscope.

5.2.4 Immunolabelling of LR White sections for EM

This was performed using MPM-2 at 1/200 and the method described previously (2.2.5). Details of the immunolabelling method were evaluated to give a maximum signal to background combination. A preliminary experiment was performed in which 16 treatments were tested using all combinations of the following parameters: with and without MPM-2 (without MPM-2 provided a 0+ negative control to test for secondary antibody non-specific binding to the sections); with and without pretreatment with 0.5 M NH_4Cl, 1 hr before immunolabelling to reduce free aldehyde groups (BioCell technical bulletin on gold conjugates); with and without 0.5% Tween 20 added to antibody dilutants to reduce non-specific background; and dilutions of gold conjugated secondary antibody of 1/20 or 1/100. Gold particles from a constant area in prints of 5-15 unstained interphase cells were counted in nuclei, cytoplasm and chloroplasts and compared. Prints were counted without knowing the treatments. Based on these tests, it was determined that 1/20 was the best dilution for the secondary antibody, and subsequent tests were performed using this dilution, and that incubating in 0.5 M NH_4Cl or using Tween in the antibody dilutants were not necessary in these experiments.

5.2.5 Analysing EM results

The apical notch region was surveyed on each section under low magnification (2,000-5,000 X) and mitotic cells were located. Therefore, the cells to be studied were chosen before the degree of immunolabelling was visible. Mitotic stages were identified as in Fowke and Pickett-Heaps (1978) and Steer (1984). For example, prophase cells were identified by nuclear shaping and the close association of microtubules with the nuclear envelope. Any cell with a phragmoplast was considered to be in telophase. In order to have an adequate area to analyse, EM micrographs were taken at magnifications of 8200, developed and printed onto 8x10 inch photographic paper. Gold particles were identified using a dissecting microscope to unambiguously distinguish them from similarly sized ribosomes. For the data on levels of labelling on cells in different stages of the cell cycle, areas on the micrographs were measured by a compensating planimeter, calculated in um^2 and the degree of immunolabelling was expressed as the number of gold particles per um^2. In order to calculate the area corresponding to the cytoplasm, the areas of the organelles were calculated separately, and subtracted from the total area of the portion of the cell visible in the photograph.

5.3 Results

5.3.1 MPM-2 staining of pine cells

Figure 5.1 presents results from MPM-2 staining of isolated cells and methacrylate sections from *Pinus radiata* root tips. These results illustrate the typical pattern of MPM-2 staining of higher plant cells. Figures 5.1a and 5.1b show examples of mitotic cells that are heavily stained with MPM-2 throughout the cytoplasm in contrast to interphase cells that have less intense staining restricted to the nucleus. Cells in telophase have noticeably less immunolabelling with MPM-2 (Fig. 5.1c), and the phragmoplast is not stained. Even though the overall staining patterns are the same as those reported in angiosperm and white spruce cells (Binarova et al. 1994), there are some differences in that kinetochores are not specifically stained (eg Fig 5.1a and 5.1b) and telophase nuclei (Fig 5.1c) are not as strongly stained as reported in white spruce embryo cells.

Metaphase pine cells in methacrylate sections were consistently very heavily stained with MPM-2. This staining dropped off during anaphase and was not present in telophase cells (Fig. 5.1d). Nucleoli were not stained.

5.3.2 MPM-2 staining of *Marchantia* tissue in methacrylate sections

Figure 5.2 presents results from MPM-2 staining of *Marchantia* gemmling methacrylate sections from the notch region. Interphase nuclei in section show a prominent staining of the nucleus around the large unstained nucleolus, giving a doughnut-like appearance. Mitotic cells had a uniformly strong immunolabelling with MPM-2. *Marchantia* cells are very difficult to stain with DAPI even with very high concentrations of this DNA dye (see section 5.2.2). Even so, one metaphase plate is prominently stained, and chromosomes are faintly stained in another (possibly) anaphase cell; these cells coincide with cells with high MPM-2 stain (Fig. 5.2). No further detail could be observed in these light micrographs, therefore the immunoelectron microscopy study was undertaken.

5.3.3 Conventional TEM of *Marchantia* tissue

The apical notch region of the young *Marchantia* gemmlings represents this primitive plant's meristematic region (Fowke and Pickett-Heaps 1978) and contains small cells with small vacuoles (Fig. 5.3). Interphase cells have round nuclei and large nucleoli (Fig. 5.3).

A prominent feature of *Marchantia* cells in prophase is that the nucleus is surrounded by microtubules and shaped like a spindle (Fig. 5.4a). At each end of the shaped nucleus is the polar organizer, an area consisting of amorphous electron dense material (Fig. 5.4b and Fig 2 in Fowke and Pickett-Heaps 1978) and surrounded by rough and smooth endoplasmic reticulum and marked by converging microtubules.

Marchantia cells in metaphase display characteristics described previously (Fowke and Pickett-Heaps 1978; Steer 1984) such as diffuse, but localized kinetochores (This is more obvious in an LR White section of a metaphase cell described below).

Figures 5.5a and 5.5a' show that a large amount of endomembranes is associated with the spindle in these plants both at the poles and also among the spindle microtubules.

A section of a cell in anaphase, depicted in Figures 5.6a and 5.6a' is another example of spindle microtubules closely associated with endomembranes; in this case microtubules terminate in an area close to endoplasmic reticulum near the poles.

A cell in very late anaphase is shown in Figure 5.7a and 5.7a'. Note the accumulation of membranes at the poles, some of which forms the new nuclear envelope surrounding the chromosome material. Long interzonal microtubules can be seen in this cell. In late telophase, the nuclei are completely surrounded by nuclear envelopes, the nucleoli have reformed, and microtubules and vesicles of the phragmoplast are easy to see (Fig. 5.8).

5.3.4 MPM-2 immunolabelling of *Marchantia* tissue in LR White sections for TEM

Table 5.1 presents a summary of results of the number of gold particles per μm^2 for different cell parts at different stages of the cell cycle. Note that the degree of staining of the

nucleus increases from interphase to prophase and that in both cases it is noticeably higher than the cytoplasmic staining. This reflects what we have seen in the methacrylate sections (Fig 5.2). In metaphase and anaphase, the level of stain in the cytoplasm in metaphase and anaphase appears to be higher than the interphase or prophase cytoplasm. This as well is similar to what we have observed with fluorescence microscopy. Even though the interphase nucleus always has a markedly higher level of label (4 gold particles per μm^2) than the interphase cytoplasm (0.5 gold particles per μm^2), the level of nuclear labelling increases dramatically sometime during the beginning of prophase (to 10 gold particles per μm^2). Condensed chromosomes are more highly labelled during metaphase and anaphase although there is considerable variation. The level of MPM-2 labelling decreases during anaphase and telophase.

Figures 5.9 to 5.12 show examples of MPM-2 staining of cells used to collect the data presented in Table 5.1. Methods for immunolabelling involving no post fixation with osmium and the use of hydrophilic resins are not as damaging to most antigens as are conventional methods (Bendayan et al. 1987), but the ultrastructural preservation of membranes especially is compromised. (Compare the chloroplast membranes in Figure 5.9 with those in Figure 5.8, for example). The nucleoplasm area is defined by the absence of ribosomes in this material prepared without osmium (Fig. 5.9, arrows, nuclear envelope). Noticeably more gold particles (Figure 5.9 arrowheads) are present in the nucleus compared to the cytoplasm in this interphase cell. The polar organizer present at prophase loses its osmophilic appearance without osmium postfixation and appears as an area of focussed microtubules (Fig. 5.10). Metaphase chromosomes have diffuse (as opposed to structured) kinetochores but these are localized to one portion of the chromosome (Fig. 5.11). Although the chromosomes are labelled, few or no gold particles are attached to the spindle

Table 5.1 Summary of MPM-2 immunolabelling of *Marchantia* cells (gold particles/µm^2)

stage	nucleus	chromosomes	cytoplasm
interphase	4.0 (1.3)*		0.5 (0.2)
prophase	10.4 (2.5)		1.9 (1.1)
metaphase		14.73 (7.5)	3.8 (2.4)
anaphase		9.8 (3.6)	2.6 (0.8)
telophase	5.9 (1.2)		1.2 (0.3)

* Numbers in brackets are values for one standard deviation.

microtubules.(Fig. 5.11). The kinetochore regions were not labelled in the few examples observed. In telophase, the nuclei and to a lesser extent the developing nucleoli have the MPM-2 phosphoepitope (Fig. 5.12). No labelling of the reforming nuclear envelope or microtubules is observed (Fig. 5.12). In interphase cells with large numbers of microtubules, little MPM-2 staining is associated with these microtubules (not shown).

No polar organizer out of the over fifty prophase cells surveyed was labelled at levels higher than the surrounding cytoplasm (for an example see Fig. 5.10). I also used confocal microscopy to optically section an MPM-2 stained methacrylate section of a prophase cell and found no staining at either polar organizer (not shown).

Negative controls using no primary antibodies had very low levels of label to the point that often a gold particle could not be found on which to focus when scanning these sections (not shown).

In summary, there was not noticeably more MPM-2 immunogold labelling at any area in which microtubules are nucleated or organized (the nuclear envelope, the polar body, the phragmoplast, or the kinetochore) compared to the surrounding cytoplasm. More generally, patterns of MPM-2 staining in both the conifer and the hepatic bryophyte reported here, are similar to those reported in angiosperms earlier, with higher levels and wider distribution of the phosphoepitopes recognized by this antibody during mitosis.

Figure 5.1. Isolated cells and a methacrylate section of *Pinus radiata* root tips stained with MPM-2, an antibody raised against HeLa mitotic cells. **a-d** staining with MPM-2; **a'-d'** corresponding nuclei stained with DAPI. All micrographs at the same magnification,
bar = 20μm.

a. MPM-2 staining of isolated cells of *Pinus radiata*. A prometaphase cell (pM) and metaphase cell (M) have high levels of stain.

b. MPM-2 staining of isolated cells of *Pinus radiata*. A metaphase cell (M) has noticeably higher levels of stain than neighbouring interphase cell.

c. MPM-2 staining of isolated cells of *Pinus radiata*. Arrowheads mark the same telophase cell in **c** and **c'**. Note the levels of staining in this telophase cell are similar to the neighbouring interphase cell.

d. MPM-2 staining of a methacrylate section of *Pinus radiata*. Note that MPM-2 staining of interphase cells is restricted to the nuclei, but the nucleoli are not stained; that staining of metaphase cells (M) is intense compared to staining of an anaphase cell (A); and that there is no staining associated with the phragmoplast in a telophase cell (T).

d'. Arrows indicate cells labelled in **d.**

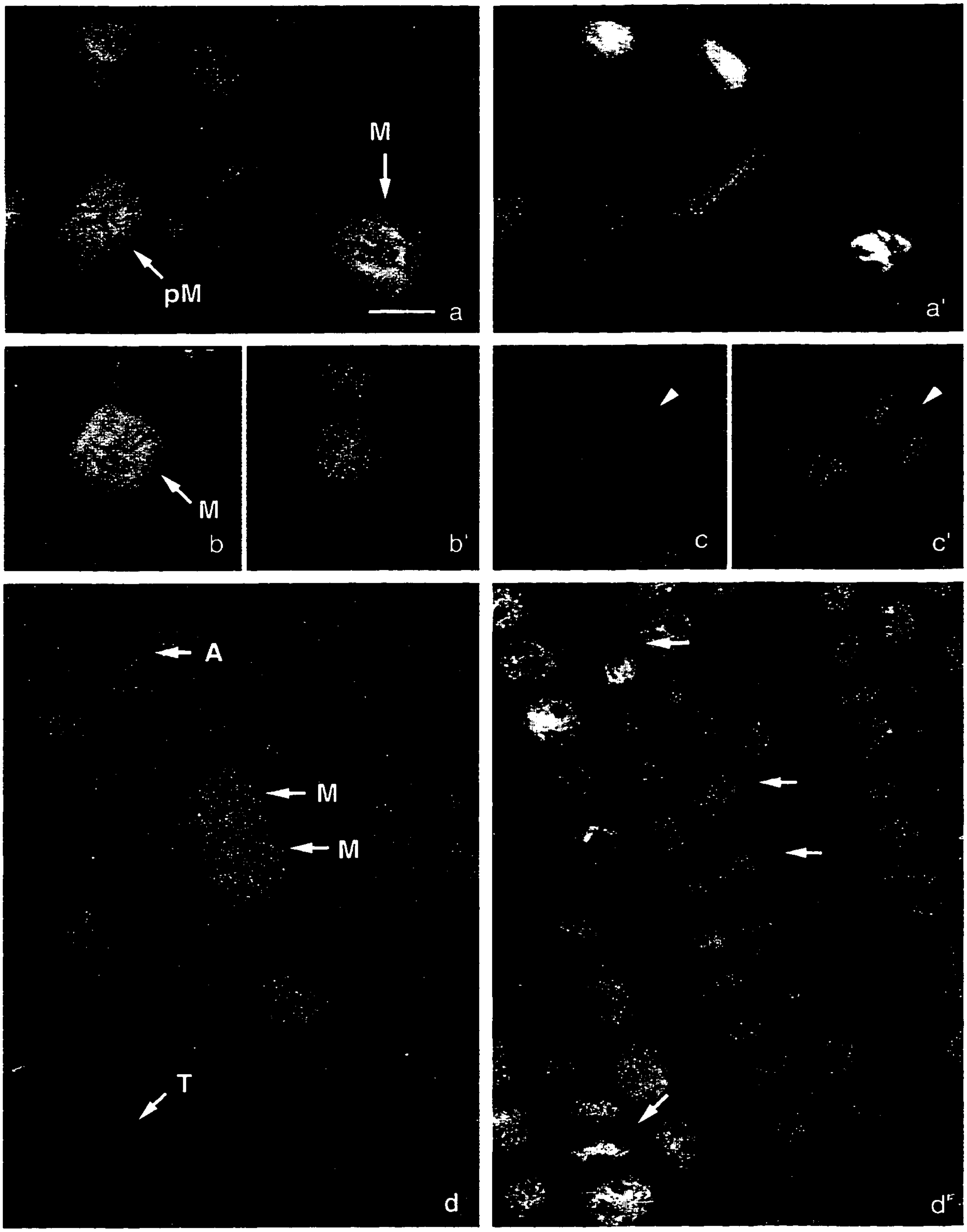

M
pM
a
a'
M
b
b'
c
c'
A
M
M
T
d
d'

Figure 5.2. A methacrylate section of the apical notch region of a *Marchantia polymorpha* gemmling stained with MPM-2, an antibody raised against HeLa mitotic cells. **a** staining with MPM-2; **a'** the same section stained with DAPI to visualize nuclei. Bar = 20μm.

a. Two dividing cells, a metaphase cell (M) and anaphase cell (A), are conspicuously highly stained throughout the cell. MPM-2 staining of interphase cells is restricted to the non-nucleolus part of the nucleus (arrowhead).

a'.. Arrows indicate cells labelled in **a.**

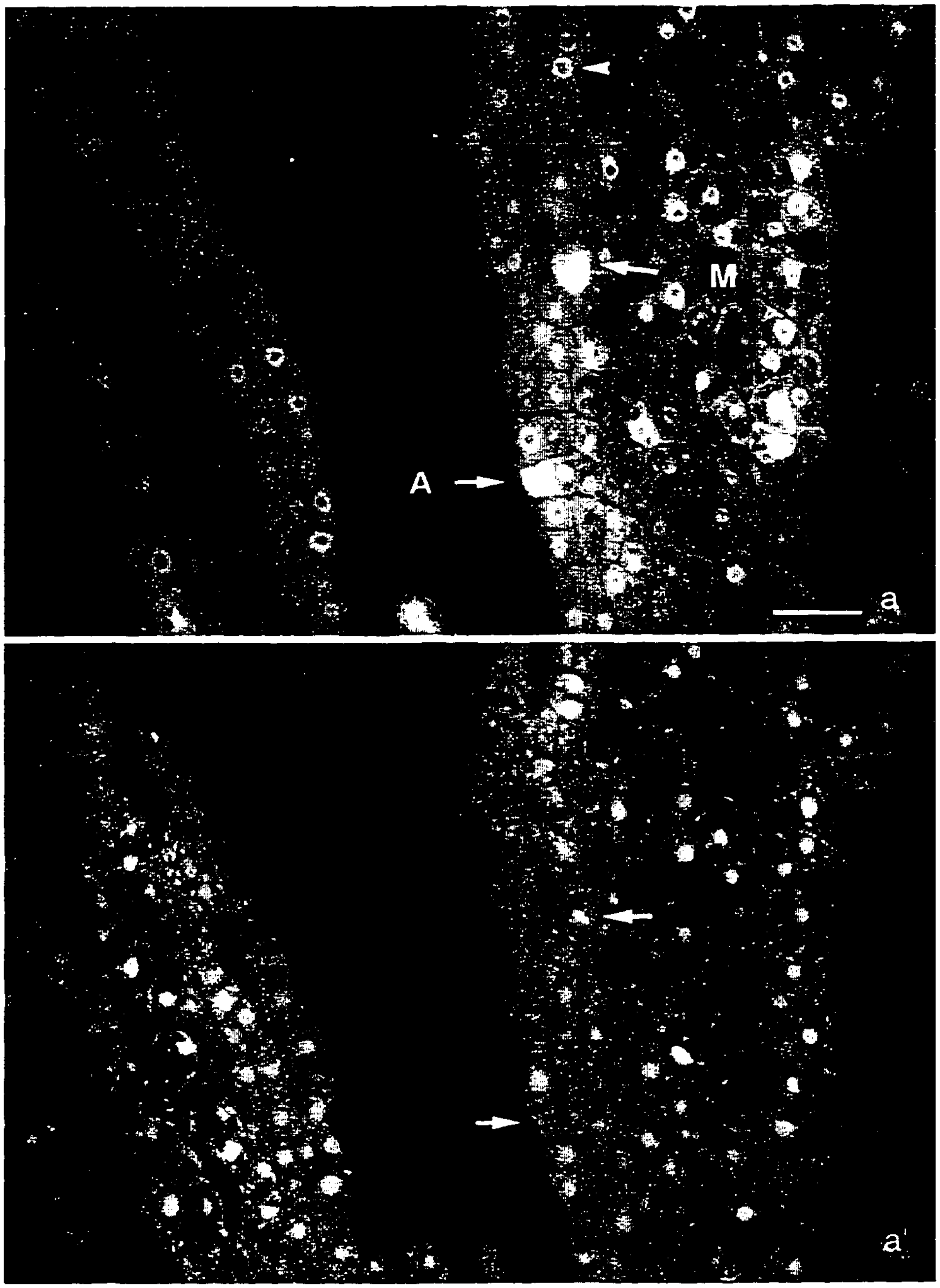

M
A
a
a'

Figure 5.3. A low magnification transmission electron micrograph of an Araldite section of a *Marchantia polymorpha* apical notch region. An arrow indicates a nucleus with a very prominent nucleolus (N) surrounded by cytoplasm containing small vacuoles. Arrowheads point to chloroplasts. Bar = 5μm.

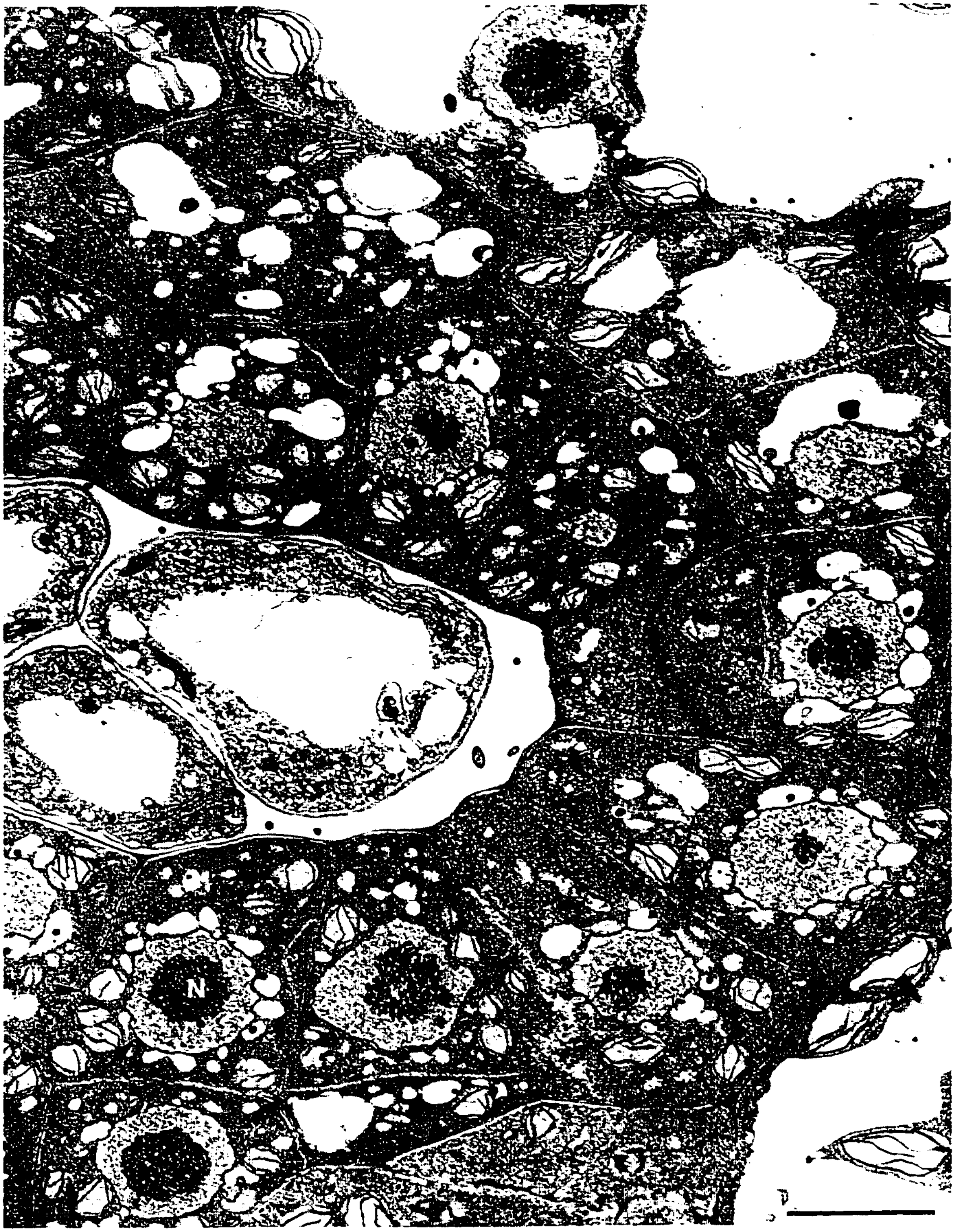

N

Figure 5.4. A transmission electron micrograph of an Araldite section of a *Marchantia polymorpha* cell in prophase.

a. A view of the whole nucleus illustrating its fusiform or spindle shape. Bar = 1μm.

a'. An enlarged view of the area outlined in black in **a**. Arrowheads indicate converging microtubules; an arrow points to a polar body, which is a putative MTOC. Bar = 0.5μm.

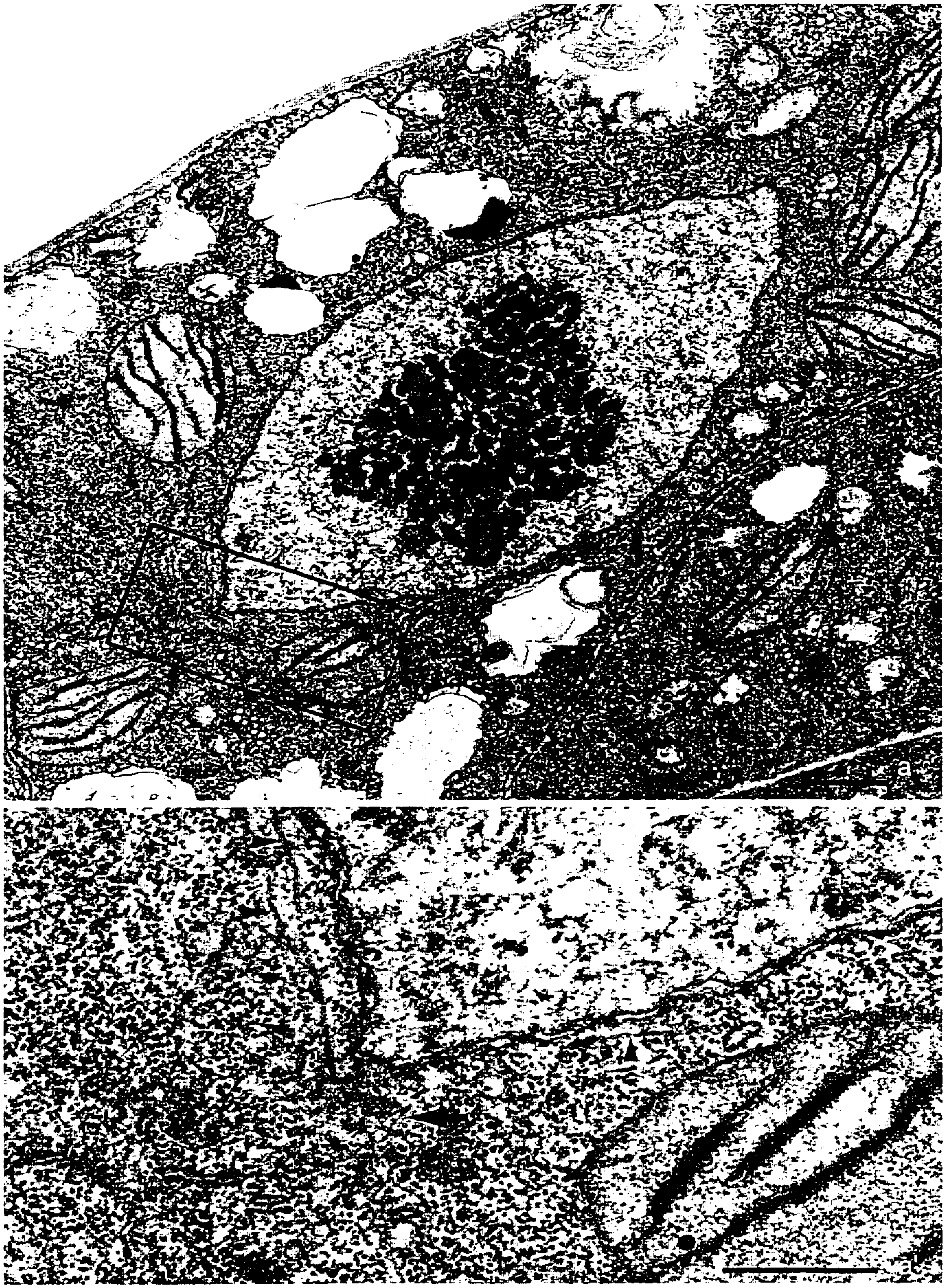
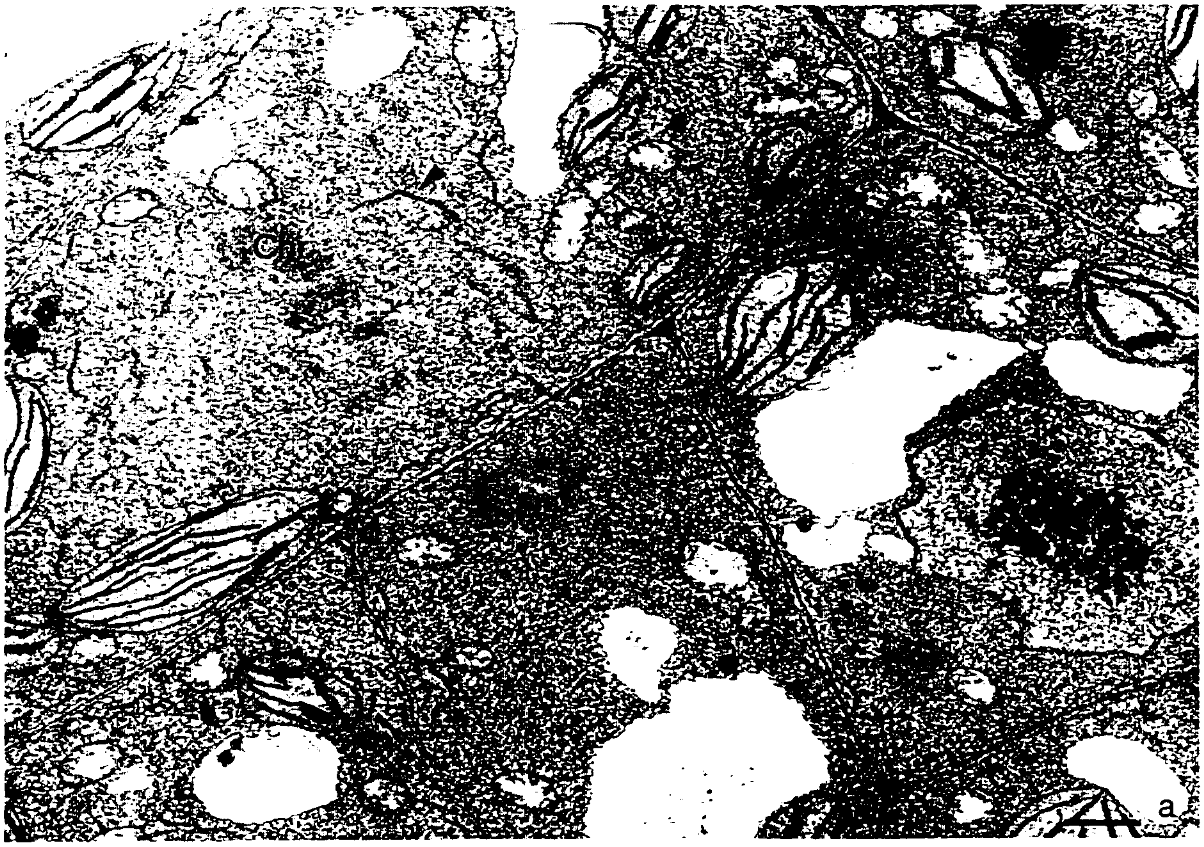

a

Figure 5.5. A transmission electron micrograph of an Araldite section of meristematic area of a *Marchantia polymorpha* apical notch, which includes a cell in metaphase. Bars = 1μm.

a. Two cells in division. Arrowhead indicates part of the extensive endoplasmic reticulum that loosely surrounds the spindle area in the metaphase cell. Chr indicates a sectioned chromosome. Note the prominent endoplasmic reticulum at the spindle poles (arrowhead).

a'. An enlarged view of the chromosomal area of the cell in metaphase in **a**. Endoplasmic reticulum (arrowheads) is evident both in the polar regions and throughout the spindle.


Ch
a

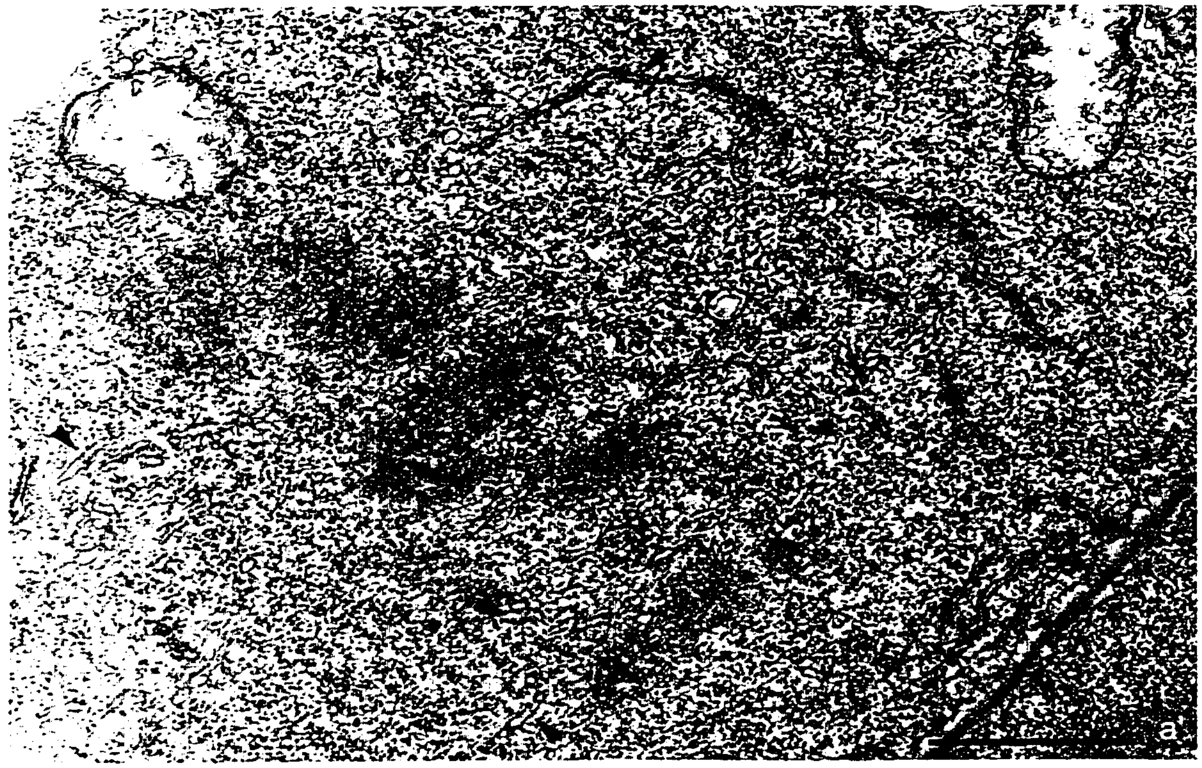
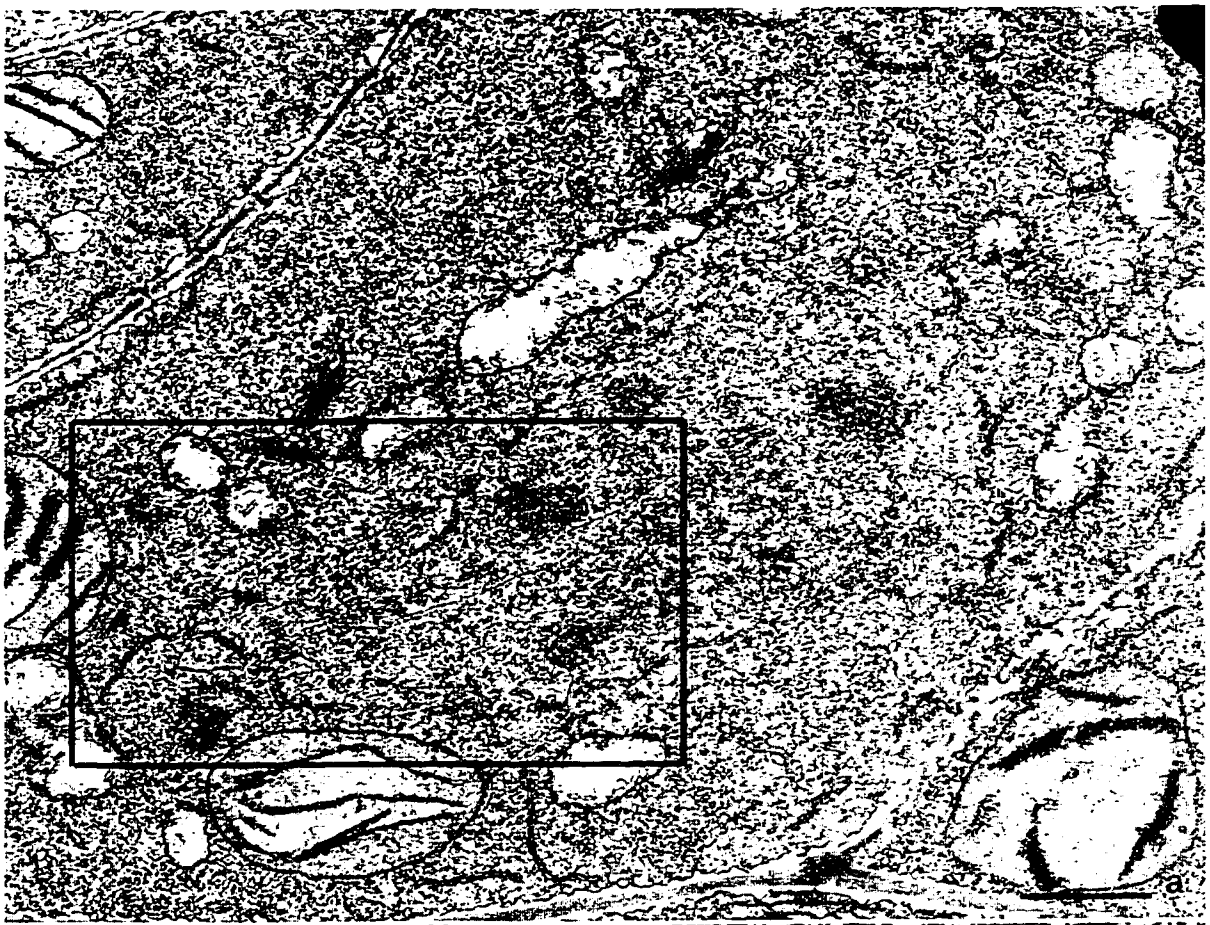

a

Figure 5.6. A transmission electron micrograph of an Araldite section of a *Marchantia polymorpha* cell in anaphase. Bars = 1 µm.

a. A view of most of the dividing cell.

a'. An enlarged view of the area outlined in black in **a**. Arrowhead indicates one of a group of microtubules that terminate in an area rich in endoplasmic reticulum, a neighbouring part of this membrane system is indicated by a double arrowhead. Chr labels a section of one chromosome.


a

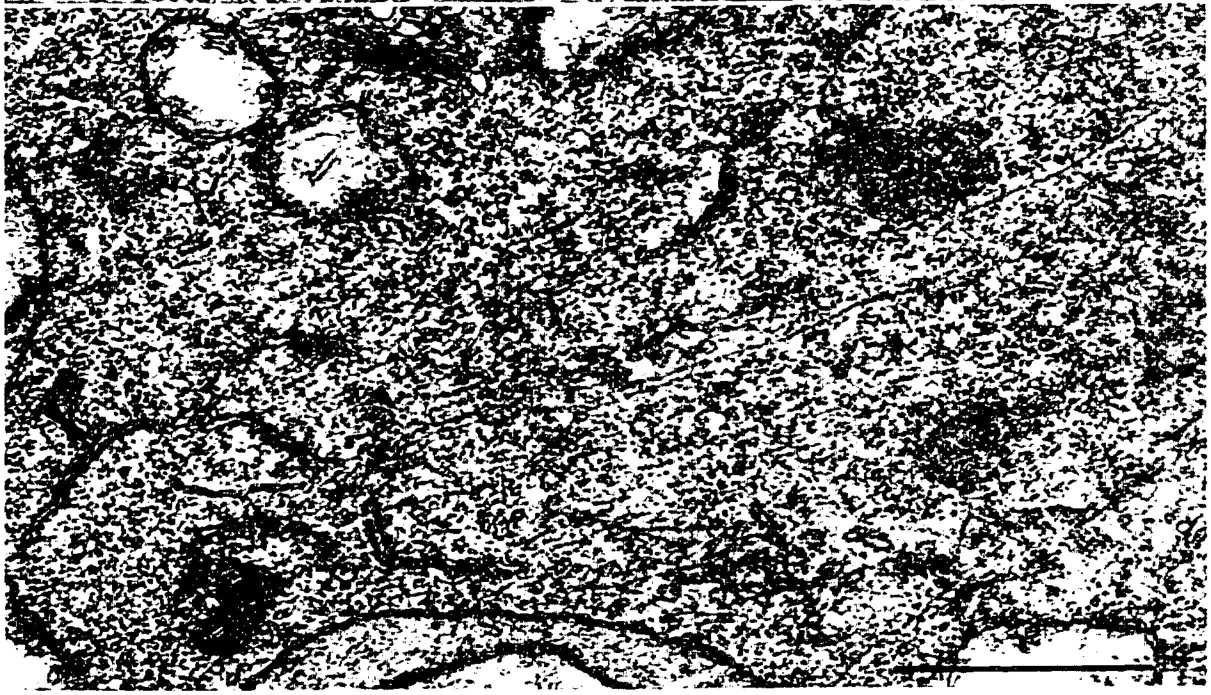


Figure 5.7. A transmission electron micrograph of an Araldite section of a *Marchantia polymorpha* cell in late anaphase/ early telophase. Bars = 1 µm.

a. A view of most of the cell. Only one reforming nucleus (large arrows) is visible; the other is out of the section. Note the prominent involvement of endoplasmic reticulum at both poles.

a'. An enlarged view of the reforming nucleus. Note the reforming nuclear envelope (arrowhead) and the many conspicuous spindle microtubules in this section (small arrows).

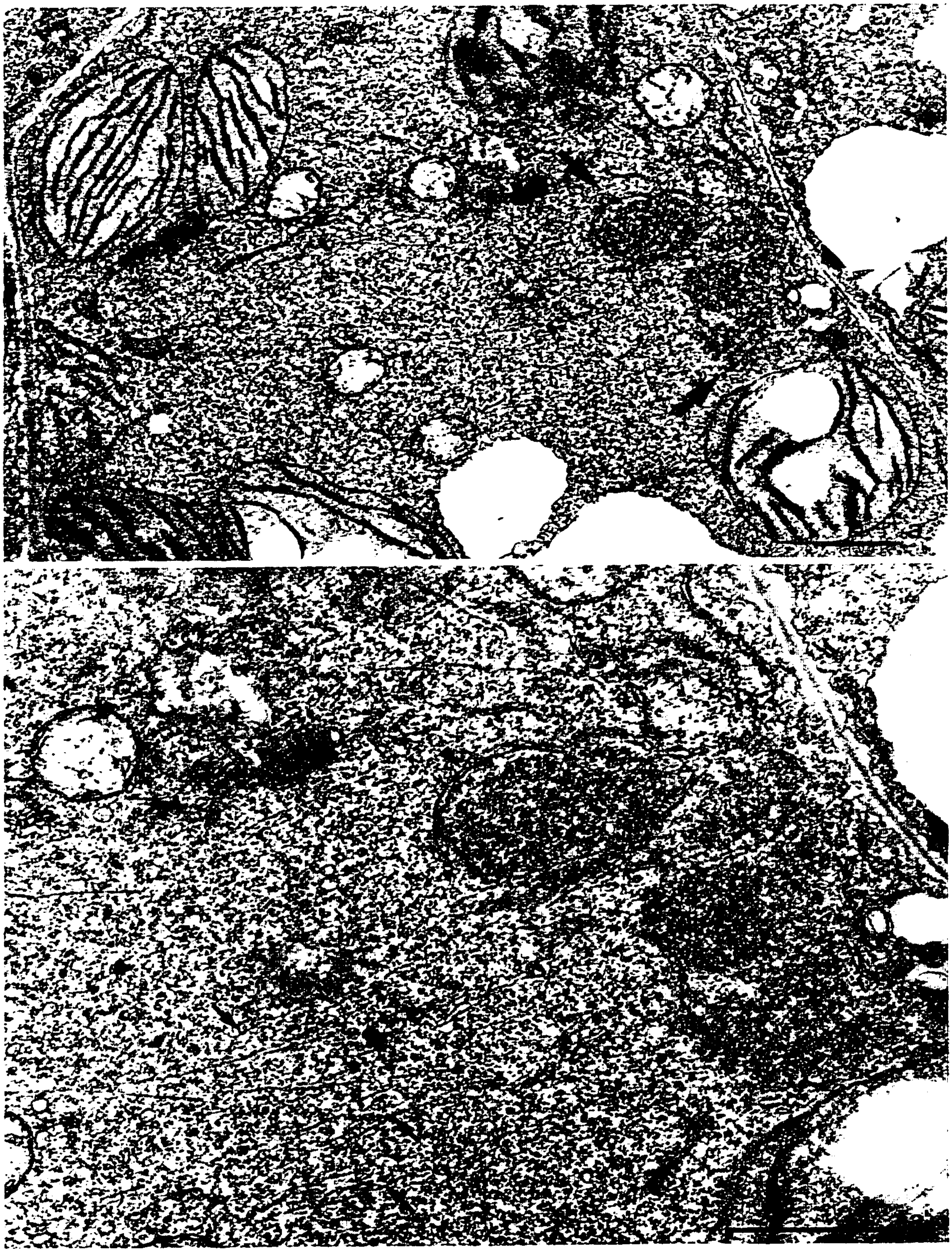
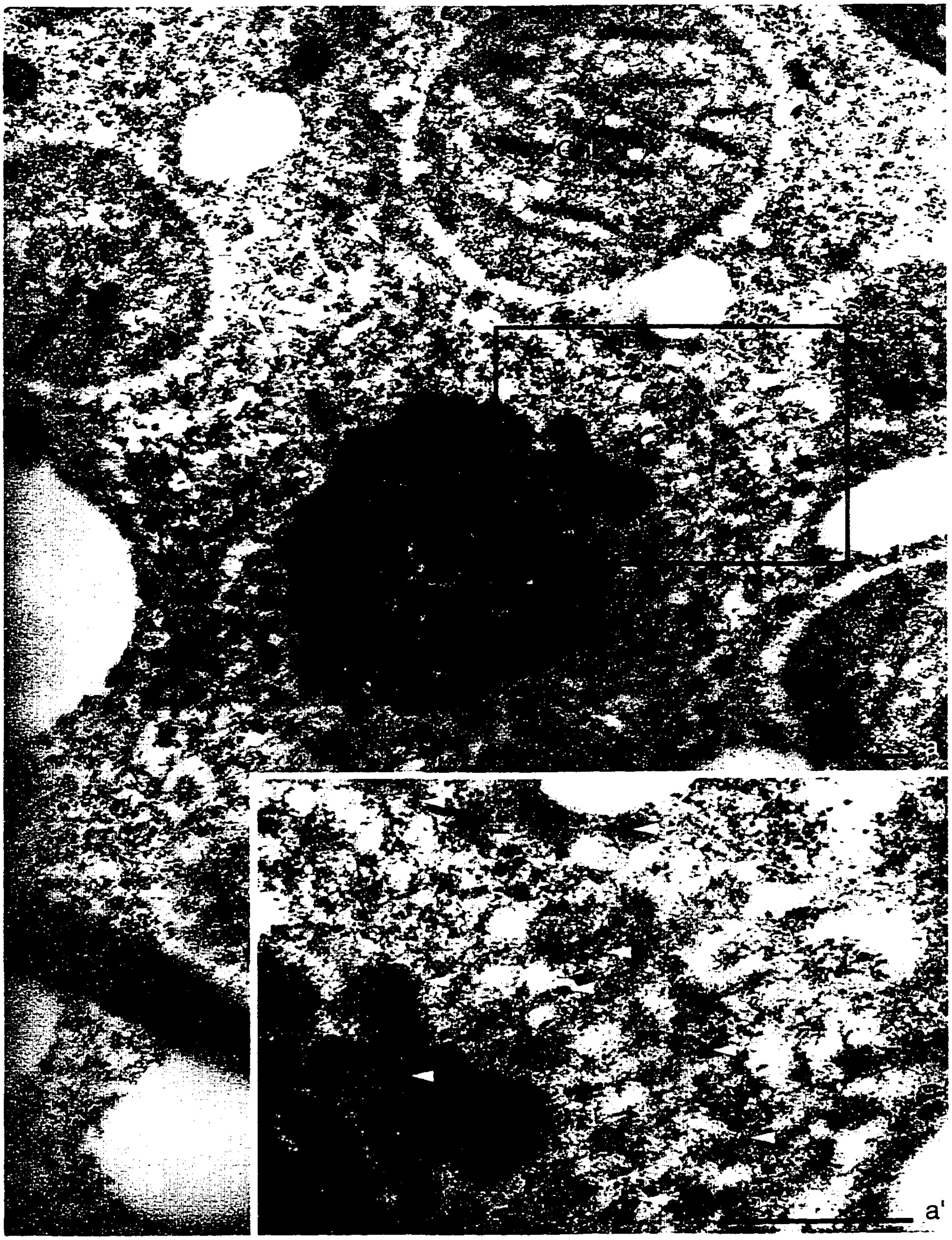


Figure 5.8. A transmission electron micrograph of an Araldite section of a *Marchantia polymorpha* cell in late telophase. The nuclear envelopes and nucleoli have reformed, and the phragmoplast, consisting of membrane bound structures (arrowheads) and microtubules (arrows) is prominent. Bar = 1 μm.

Figure 5.9. An interphase *Marchantia polymorpha* cell in an LR White section stained with MPM-2, an antibody raised against HeLa mitotic cells. Bars = 0.5μm.

a. MPM-2 staining of an interphase cell. Arrows point to one edge of the nucleus. The nuclear envelope is not distinct, nor is the chloroplast (Chl) envelope.

a'. An enlargement of the area outlined in black in **a.** Arrowheads point to immunogold particles; there are many of these particles in the nucleus, but few in the nucleolus.



a
a'

Figure 5.10. A polar organizer (arrows) close to the plasma membrane in a *Marchantia polymorpha* prophase cell in an LR White section, stained with MPM-2, an antibody raised against HeLa mitotic cells. Note the microtubules (arrowheads) converging at the polar organizer and the nuclear pores (one indicated by a double arrowhead) on the nuclear envelope. Bar = 0.5μm.

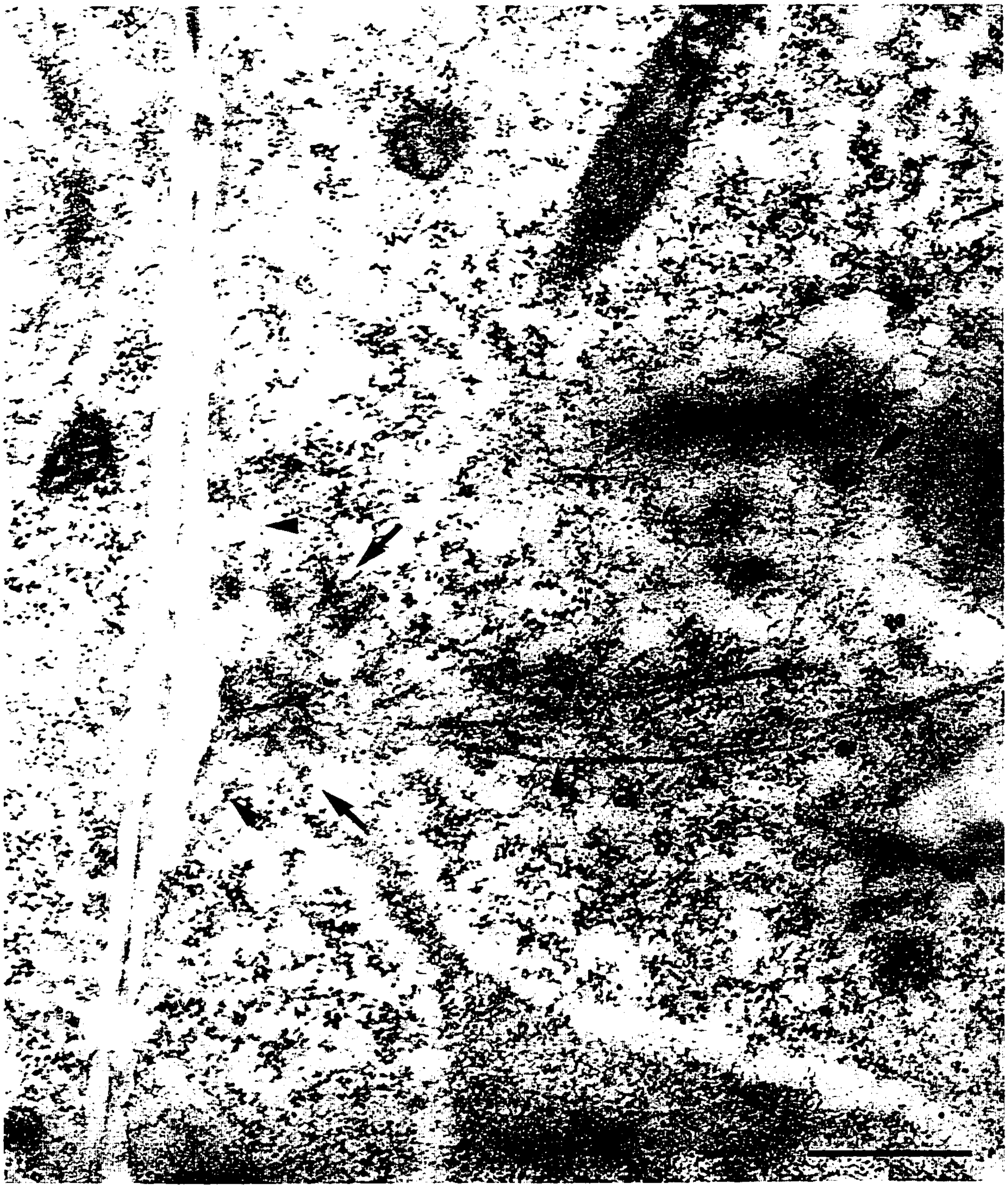


Figure 5.11. Two chromosomes in a *Marchantia polymorpha* metaphase cell in an LR White section, stained with MPM-2, an antibody raised against HeLa mitotic cells. The microtubules (larger arrowheads) are attached to the diffuse kinetochores. Although the chromosomes are labelled with MPM-2 and there is some label in the spindle area, no microtubules are labelled (gold particles indicated with small arrowheads). Bar = 0.5μm.

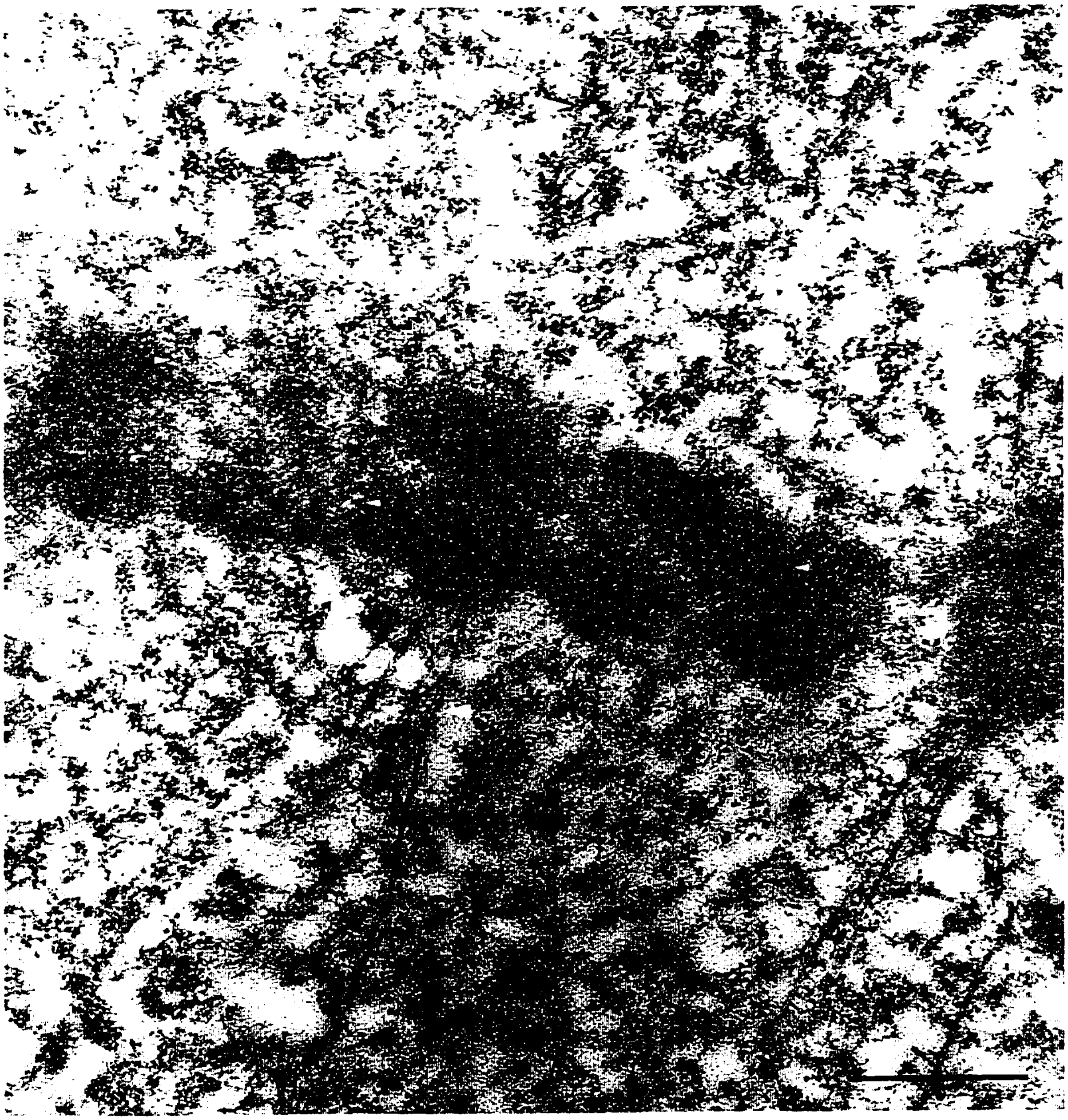
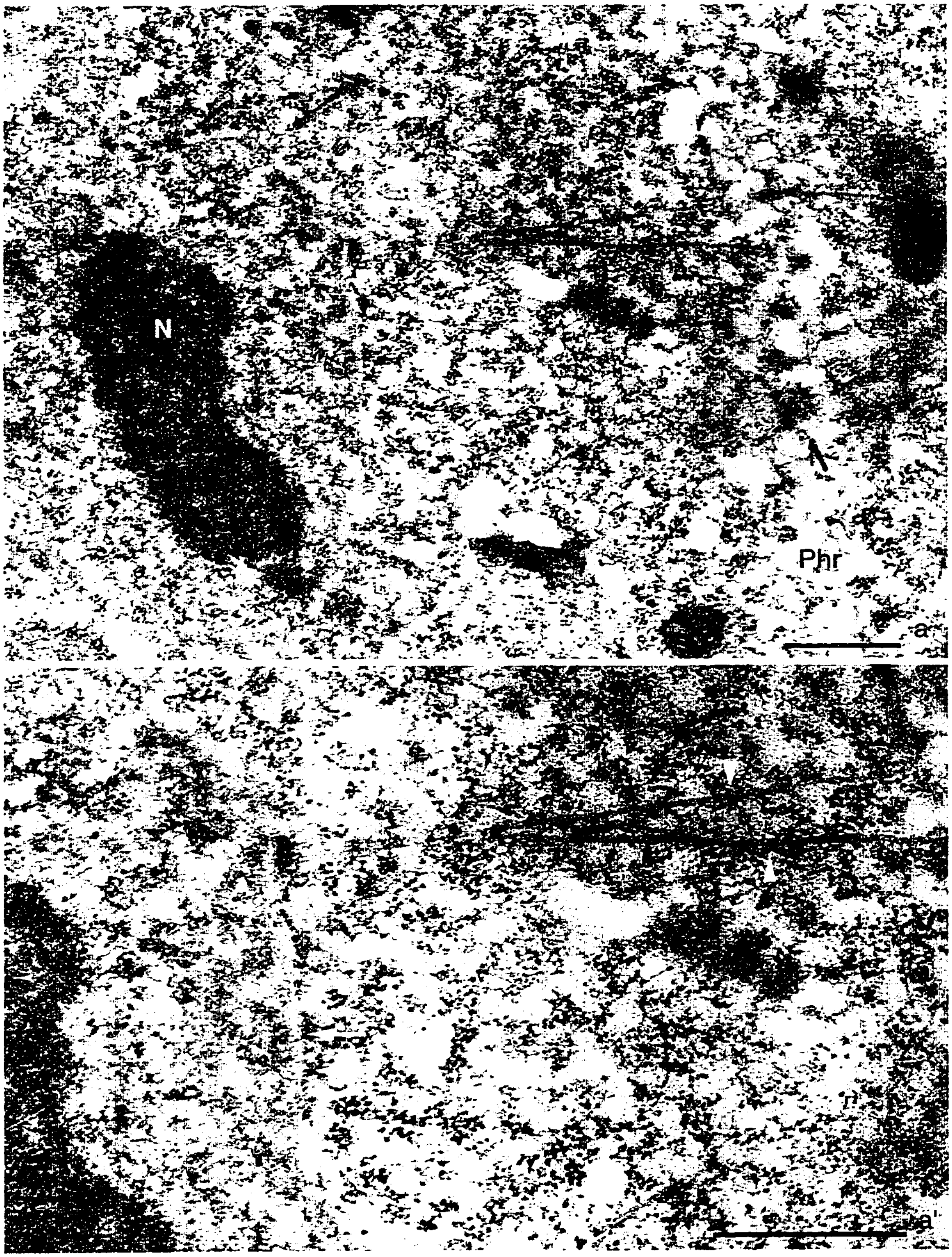


Figure 5.12. A reforming nucleus in a telophase *Marchantia polymorpha* cell in an LR White section, stained with MPM-2, an antibody raised against HeLa mitotic cells. Bars = 0.5μm.

a. MPM-2 staining of part of a telophase cell. A reforming nucleolus (N) and the phragmoplast (Phr) are visible.

a'. An enlargement of the area including the reforming nuclear envelope. At least some microtubules (larger arrowheads) seem to be attached to the reforming nuclear envelope. This telophase nucleus is labelled with MPM-2. No microtubules are labelled (gold particles indicated with small arrowheads). There are more of these particles in the nucleus, and more in the nucleoplasm than in the nucleolus.


N
Phr
a
a'

5.4 Discussion

The presence of MPM-2 labelling in pine cells that corresponded to results reported earlier for other higher plants including conifers (Traas et al. 1992; Binarova et al. 1994) confirmed that the methods of tissue fixation and immunolabelling used in this study were appropriate for this antibody. The similar MPM-2 localization to interphase nuclei and mitotic cytoplasm in *Marchantia* methacrylate sections, and also in LR White sections indicates that not only is it possible to infiltrate and embed these bryophyte gemmlings, which are only a few cells thick, with both polar and non-polar resins for EM, but also, in the former case preserve the MPM-2 epitope. Although recent divisions are evident and many examples of prophase have been found, cells in metaphase or anaphase are rare, indicating that these stages are relatively brief (see also Fowke and Pickett-Heaps 1978; Steer 1984).

The fact that no specific labelling of the polar organizer with MPM-2 was detected could be because: (1) the polar organizer nucleates microtubules, but the MPM-2 epitope is not present in this membrane associated MTOC; (2) the epitope was there, but was extracted or damaged by treatments; or (3) the microtubules are nucleated somewhere else – perhaps the nuclear envelope – and the converging microtubules result from other processes. In the last case, the nuclear envelope could act as it is believed to in higher plant cells; the converging microtubules at the polar bodies could be the result of minus end directed microtubule motors. In this case, the osmophilic polar bodies could be the accumulation of anchoring membranes and proteins. Recent experiments using brain tubulin and *Xenopus* egg extracts have shown that apparently normal spindles with microtubules converging at their minus ends can be formed by microtubule motors without an organizing centrosome (Heald et al. 1996). Furthermore, a monoclonal antibody to a dynein subunit injected into mammalian cells can interfere with the formation of bipolar spindles even in cells with centrosomes (Gaglio et al. 1997). Minus end directed microtubule motors acting in a similar fashion could explain the formation of the prophase spindle on endosperm nuclei, where microtubule prophase spindles seem to self-assemble forming two or more poles of converging microtubules (Smirnova and Bajer 1998) and the polar organization in black spruce prophase spindles where the poles seemed to lift off the nuclear surface (Wang et al. 1991). It is intriguing to speculate that the nuclear shaping that occurs in prophase in *Marchantia* may also be the result of membrane-attached microtubule motors on the nuclear envelope. The cytoskeleton acts to organize membrane organelles in all eukaryotes, and this might be another example (Allan 1995). Further work, starting with the identification of motors in these cells will be needed to determine the extent of their involvement in the well described structures of *Marchantia* prophase. If microtubule motors are responsible for the organization of the microtubules in hepatic prophase, then the polar body would not be an MTOC, but an anchoring centre. It must be emphasized that this is speculation only, based on the recent unexpected findings regarding the central role microtubule motors are believed to play in microtubule organization (reviewed in Hirokawa et al. 1998).

As was noted above, hepatic cells share many characteristics with higher plant cells including similar kinetochore structure and nuclear envelope behaviour in mitosis (Steer 1984). The temporal and spatial pattern of MPM-2 staining reported in this study is another similarity. The phosphoepitope recognized by MPM-2 was present in the interphase nucleus and increased during mitosis where it was present throughout the cytoplasm. One slight difference is the higher level of staining in anaphase *Marchantia* cells as compared to the anaphase staining of pine cells. The lack of staining of either possible MTOC in these cells – the nuclear envelope or the polar body – is perhaps another similarity to other plant cells. Even though MPM-2 does label MTOCs in animal and fungal cells (Vandre et al. 1984; Engle et al. 1988), it does not appear to label minus end nucleating sites in plant cells. It may be significant that the one MTOC in plants that has been unambiguously recognized by this antibody, the blepharoplast, is one that is not associated with membranes (Hoffman et al. 1994). Either the blepharoplast is the only MTOC that contains this antigen, or when the antigen is associated with membranes it is altered by tissue preparation methods used for immunolabelling.

The results described in this study resemble the results seen in the previous chapter. Here again, putative MTOCs are not unambiguously labelled by an antibody that clearly recognizes the centrosome in animal cells.

5.5 References

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Chapter 6. Discussion and future prospects

The objective of this study was to determine if some of the features reported in animal cytoskeletons– posttranslational modifications in tubulin, γ-tubulin and other centrosomal constituents– are present in meristematic plant cells. This section includes a discussion of the methods used and a general discussion of some of the results described in detail in earlier chapters with proposals for future research.

6.1 Discussion of tissue preparation methods

In especially the last ten years, immunological methods have been used both to examine the components of MTOCs and to biochemically dissect cell cycle events in organisms from different phyla. This immunological approach is particularly appropriate because mitotic cells are at any one time only a small proportion of the population of cells and important mitotic proteins may be present in only a small number of copies, so that classical biochemical methods would give impossibly small yields (Kuriyama 1992; Rose et al. 1993).

Fixation and preparation of tissues for immunolabelling of any kind is a trade-off between immobilizing the cell components and having the cells penetrable to antibodies. This is accomplished routinely in plant tissue by fixing in formaldehyde-based fixatives, with microtubule stabilizing agents present if the cytoskeleton is to be studied, and then extracting with detergents. Fixation with aldehydes is necessary for plant cells partly to enable the cell to withstand the harsh methods used to digest or otherwise permeabilize the cell wall (Wick 1993). Extracting with detergents is necessary not only to make the cell penetrable to antibodies but also to reduce autofluorescence, which is a conspicuous feature of plant cells. Two general types of fixation methods for immunolabelling were used in this study: (1) formaldehyde fixation followed by cell wall digestion and then detergent extraction for isolated root tip squash cells, and (2) aldehyde fixation followed by dehydration and embedding in resins for sectioning, either in methacrylate for fluorescence microscopy or a hydrophilic resin appropriate for TEM studies. Both types of methods provide material with advantages and disadvantages. For immunofluorescence studies, we can optically section whole, isolated cells to understand three-dimensional structures, while the methacrylate sections allow us to survey a large number of cells within a tissue context. The isolated cells allow us to observe plant tissue that has not necessarily been exposed to alcohol dehydration, while methacrylate sections allow us to use tissue that has not been extracted with detergents. Methacrylate sections are ideal for testing a number of antibodies. Because the methacrylate is removed immediately before the sections are used, exposing "fresh" tissue, sections can be stored until needed and the same tissue can be tested over a period of time.

Fixation of animal cells often relies on the use of methanol alone. The difference in fixation methods may contribute to some of the discrepancy in immunolocalization results reported in animal cells and those reported in plant cells. A number of recent reports about localizing proteins in plant cells emphasize the importance of methanol as a fixative for various MTOC components. This is often presented as a way of uncovering the epitope. For example, cold methanol fixation seems to be important for preservation of MPM-2 and MAP (microtubule associated proteins) epitopes in one study (Smirnova et al. 1995). 6C6 localization seems to depend on at least a post-fixation with cold methanol (M. Vantard, personal communication). Methanol treatment was also necessary for the visualization of γ-tubulin in one report (Nováková et al. 1996). This reliance on methanol for the preservation or recognition of at least the γ-tubulin epitope is not absolute, however. One lab reported that although fixation with methanol was best, formaldehyde fixation with a methanol post-fixation was adequate, but that treatment with methanol was required to uncover the γ-tubulin epitope (Nováková et al. 1996). Liu et al. (1993) states that formaldehyde enhanced staining compared to a methanol fixation using their γ-tubulin antibody. We routinely tested each antibody on tissue prepared both with and without a cold methanol treatment, as well as other factors in order to assess staining under a variety of conditions.

Another reason to use a variety of methods to prepare material for immunological studies is because of the danger of destroying an epitope by preparation methods (Larsson, 1988). Cryosections and cells released from digested and squashed tissue have the advantage of using material that has not been dehydrated with solvents. Because plant MTOCs are intimately associated with membranes, the nuclear envelope and the ER which is found in the polar organizers and closely associated with spindles, it is likely that at least some of the proteins necessary for this process are integral proteins. There is a danger that the dehydration necessary for preparation of material for all types of resin embedding could destroy epitopes on these proteins either by extraction or by changing polar and non-polar regions. Some reports indicate that material which has not been dehydrated is preferable for some studies. Hause et al. (1995) state that cryosectioning is superior to PEG embedding for the MPM-2 staining of pollen and microspores. Similarly, in an earlier study, squashed root tip cells showed kinetochore MPM-2 staining and embedded tissue did not (Binarova et al. 1994). In both cases, the loss of the epitope is attributed to the embedding procedure. Although any immunotechnique that uses detergents or methanol to permeabilize membranes is also potentially a problem (Melan and Sluder 1992), material as thick as a plant cell that is not extracted with detergents is almost impenetrable with any visualizing method. There is even some evidence that the apparent location of the antigen can occasionally be affected by the choice of fixative. Kuriyama (1992) reports that a monoclonal antibody raised against *Dictyostelium* MTOCs reacted to the whole length of Chinese hamster ovary cell microtubules in cells fixed in glutaraldehyde but to the centrosome only in those fixed in methanol. This points again to the importance of using a variety of fixation methods either within one lab or between labs to confirm results. The two tissue preparation methods used in this study, embedding methods in which blocks of tissue were dehydrated but not extracted with detergents and methods to prepare isolated whole cells that use detergent extraction but not dehydration, gave similar results for some but not all components investigated. The results using antibodies to the posttranslationally modified tubulins as well as the results using antibodies to α- or β-tubulins and MPM-2 were unaffected by preparation method. Those results based on centrosomal components were more problematic. Results reported above for all three were obtained using whole, isolated cells. Neither γ-tubulin nor centrin was detected on methacrylate sections. However, γ-tubulin has been detected in our study in LR White sections, and both have been detected by other labs in LR White sections in distributions similar to that reported in whole cells (Vaughn and Harper 1998).

Although there is certainly a concern about tissue preparation methods, it is not appropriate to abandon studies using fixed material. Most of the evidence that we have about cell anatomy has been based on studies using fixed material and, except for the unexpected dynamic nature of microtubules in plant cells (that static images cannot reveal except in the most indirect ways) it is remarkable how little of this information needs to be revised as the result of recent work on living cells. For example, Valster and Hepler (1997) studied the distribution of actin microfilaments and microtubules in living *Tradescantia* cells, and found very similar distributions of both elements in the cytoskeleton of dividing cells to those reported earlier, based on fixed material. In fact, even for the aldehyde sensitive microfilaments, the structures reported in this study and one 10 years earlier (Traas et al. 1987) were essentially the same, including a cortical array, a net around the spindle with some spindle localization, and phragmoplast staining. To be sure, the study on living cells allowed real time visualization of the effects of caffeine on plant cytokinesis, but again, the results were essentially the same as those reported earlier.

Other significant factors in immunolocalization of cytoskeletal components are not as controversial. Both the selection of an antibody and the visualization method are important for these studies. For example, only two of a number of antibodies raised against γ-tubulin gave positive results and the use of a confocal microscope was crucial in the interpretation of these results. Even though the method is very time-consuming, immunoelectron microscopy is also sometimes necessary to achieve sufficient resolution.

6.2. Plant posttranslational modifications to α-tubulin

The presence of acetylated tubulin in all microtubule arrays in pine cells, and in at least some arrays in all conifers tested and one bryophyte species indicates that these plant groups have the enzymes responsible for acetyltransfer and deacetylation. The abundant presence of acetylated tubulin in mitotic conifer cells contrasts sharply with its very limited distribution in angiosperm cells examined and indicates that gymnosperms and angiosperms may differ considerably. The fact that even in the presumably labile population of mitotic microtubules, acetylated microtubules were present indicates that the enzyme responsible for the modification must act rapidly. The presence of acetylated tubulin in what is believed to be relatively stabilized portions of the mitotic and cytokinetic arrays indicates that the antibody to this modification could be useful for mapping microtubule dynamics in conifer cells.

When plant cells were probed with antibodies to detyrosinated tubulin and Δ-2 tubulin, no microtubules were stained (Chapter 3) or only small speckles along the microtubules were stained (Smertenko et al. 1997). In the results reported in Chapter 3, the negative results were confirmed by the fact that the epitope recognized by the antibodies to detyrosinated and Δ-2 tubulin could be created on the sections or whole cells. The lack of these modifications or the presence of them in very small quantities indicates that the enzymes responsible for them may not be present in plant cells, or may be present in only very limited quantities. This suggests that the removal of C-terminal amino acids in α-tubulin is not a marker of stabilized microtubules in the same way that it seems to be in a number of animal cells. As discussed above (3.5) this may reflect some differences between plant and animal cells in cellular morphogenesis and Golgi distribution.

Further studies to determine the distribution of these two modifications in other plant cells, including meristematic cells of other plant species and differentiating cells, will assist in our understanding of both the function of these modifications in all cells and their role in microtubule dynamics in plant cells.

As mentioned in Chapter 1, other posttranslational modifications to tubulins exist. A number of kinases have been shown to phosphorylate a number of sites in both soluble and polymerized tubulin and extensive glutamine and glycine chains can be covalently attached to a glutamine residue near the C-terminus of both α- and β-tubulins incorporated into microtubules (reviewed in MacRae 1997). While there are no antibodies that recognize the phosphorylation of specific sites on tubulin, antibodies have been raised that recognize both polyglutamylated and polyglycylated tubulin. Polyglutamylated tubulin has been found in a variety of organisms including cultured tobacco cells (Smertenko et al. 1997) where it was found in all microtubule arrays and in both α- and β-tubulins. This modification seems to influence MAP binding in animal cells, including the motor protein kinesin (reviewed in Ludueña 1998). It would be very interesting to see if polyglutamylation is present in other plant cells, and if other plant cells have it in all arrays. Although polyglycylation has so far been found only in axonemal microtubules including those in protists and vertebrate sperm (reviewed in Ludueña 1998), it may be induced by the interaction of microtubules with membranes (reviewed in MacRae 1997), and therefore may be present in plant cells.

Although it has been postulated that one function of posttranslational modifications of tubulin may be to modulate microtubule interactions with MAPs (Ludueña 1998), there have been few studies to see if there are any similarities between the distribution of posttranslationally modified tubulin and MAPs. Few plant MAPs have been identified, let alone antibodies raised against them. However, studies in animal neuronal cells, rich in both MAPs (with antibodies which recognize them) and posttranslationally modified tubulin, could determine if MAPs and tubulin modifications are distributed on the same microtubule structures.

6.3 The nature of the higher plant MTOC

The nature of the MTOC in plants is poorly understood. In this study, we examined conifer and bryophyte cells for three components of animal MTOCs, and one phosphoepitope also associated with animal MTOCs. Our results and the results of other similar studies (reviewed in Vaughn and Harper1998) are one part of a process to identify microtubule organizing sites in plant cells and to understand the process of microtubule organization generally.

It is commonly understood that MTOCs nucleate, stabilize and orient microtubules (Vaughn and Harper 1998). Our understanding of this process has evolved considerably. More than ten years ago, a "search and capture" model for the formation of mitotic spindles was developed that can be summarized as follows: dynamic, unstable microtubules nucleated at the MTOC, grew in all directions and were captured by kinetochores (Kirschner and Mitchison 1986). This model explained the observations in most animal cells quite well, but was less helpful with observations of microtubules in acentriolar cells, including higher plant cells, where recognizable MTOCs are not present.

Since then, three discoveries have enriched, challenged and broadened this model, making it more widely applicable, especially to plant cells. These were: (1) the discovery that microtubule motors have a prominent role in the assembly of microtubule spindles and that this organization of microtubules is separate from the nucleation of microtubules (Heald et al. 1997); (2) the discovery that γ-tubulin is essential for the nucleation of microtubules *in vivo* (reviewed in Marc 1997); and (3) the discovery that microtubules in plant cells, even cortical microtubules, have a fast turnover rate (Hepler and Hush 1996).

The experiments that illustrate the important role microtubule motors play in the formation of microtubule arrays showed that bipolar spindles could form around chromatin covered beads in *Xenopus* egg extracts and that these spindles could form without centrosomes or kinetochores, but did require microtubule motors (Heald et al. 1996). The discovery that microtubules can be nucleated on one structure (both chromatin covered beads and centrosomes were used in the original experiments, Heald et al. 1996, 1997) and then self-organized into spindles by other processes is especially applicable to observations of plant microtubule organization. The prophase spindle is probably nucleated on the nuclear envelope, but the poles could very well be organized by a combination of plus end and minus end directed microtubule motors (Marc 1997). Similarly, at least some of the microtubules that form the phragmoplast are nucleated at the reforming nuclear envelopes (Baskin and Cande 1990) and microtubule motors are probably involved in the organization of the phragmoplast (Asada et al. 1997). One pleasing aspect to this model of microtubule organization is that the same mechanism applies to cells with centrosomes as well. In these cells there is evidence that although the centrosomes nucleate microtubules, the spindles are self organized by a combination of microtubule motors (Heald et al. 1997). The minus end directed motor is believed to tether the spindle pole to the centrosome (Heald et al. 1997).

The indication that microtubule organization in plants and animals may be the result of common mechanisms makes it even more important to determine which centrosomal components are present in plant cells and where they are located in order to "functionally dissect" MTOCs. For example, our finding that centrin is not found at the nuclear envelope, and the fact that this has been the case in other (but not all) higher plant studies (reviewed in Vaughn and Harper 1998) suggests that centrin has diverse functions in plant cells that may or may not include microtubule nucleation.

The fact that γ-tubulin is seen to be the universal component of microtubule nucleation (Oakley 1995; Marc 1997) has made staining by γ-tubulin antibodies a significant marker for nucleating sites in plant cells; and we would expect every nucleating site to be stained with γ-tubulin antibodies. However, as has been discussed above (4.4), every site of γ-tubulin staining is not necessarily a nucleating site. In the series of experiments on which the model of microtubule organization described above is based (Heald et al. 1997), the distribution of γ-tubulin is similar to the results reported in plant cells in this (Chapter 4) and other studies (eg Liu et al. 1995; Joshi and Palevitz 1996; Marc 1997): towards the minus ends of microtubules, but also along microtubules. This could be due to a high concentration of γ-tubulin in *Xenopus* egg extracts in which the experiments were performed (Gard 1994). The similar apparent distribution suggests that γ-tubulin is present in plant cytoplasm in relatively high concentrations. It may also indicate, as suggested (Marc 1997; Smirnova and Bajer 1998), that plant microtubules may be involved with the redistribution of microtubule nucleating material.

The high turnover rate for plant microtubules indicates that cortical sites nucleate cortical microtubules. The depolymerization and repolymerization of cortical microtubules has been rejected in the past as a mechanism to explain the relatively rapid reorganization of cortical microtubules in response to a variety of environmental conditions because it had been assumed that the microtubule turnover rate would not be fast enough (see Hepler and Hush 1996). The findings that at least some cortical microtubules have half times of about 1 minute clearly support the possibility that the dynamic instability of these microtubules could be an important part of their reorientation (Hepler and Hush 1996). The results in Chapter 4 of the staining of both cortical and nuclear envelope sites with an antibody to γ-tubulin is an exciting contribution to this field.

The separation of the nucleation and organization functions allows us to look for these components separately in trying to understand the concept of the MTOC as it refers to plant microtubules. The simplest form of MTOC may be γ-tubulin, which in turn may interact with cell cycle and developmental modulators such as calcium or phosphorylation (Del Vecchio et al. 1997). It is still fruitful to look for components of animal MTOCs and other MTOCs in plant cells. Besides locating components at the MTOC, there should be some investigation of what role they perform. Some components of animal MTOCs are involved in nucleating microtubules (like γ-tubulin); others appear to have different functions such as cell cycle regulation (e.g.MPM-2 epitopes); others may be involved in the duplication of the centrosome (e.g. centrin). Some components of the centrosome may be involved in the structure that organizes the nucleating material in pericentriolar material. These could include pericentrin, which is a fibrous protein always associated with centrosomes (Doxsey et al. 1994), but that has not been reported to be located in other putative MTOCs. Some components may attach to centrosomes to be divided between daughter cells (Kuriyama 1992). In plant nucleating centres we would not expect to find centrosomal components that are involved in the duplication and separation of the centrosomes nor perhaps those involved in the structural organization of the nucleating material unless it was also involved in attaching it to membranes.

From the point of view of plant cell biology, it is important to use antibodies to centrosome components to contribute to the debate regarding the nature of the plant MTOC. In the more general field of cytoskeletal regulation, it is important to understand what factors are essential in MTOCs and what is unique to each phyla. Using antibodies raised against conserved components from other organisms with structural MTOCs has been called "still the best, most promising in elucidating the nature of the amorphous higher plant MTOCs" (Del Vecchio et al. 1997).

This study is the first on the distribution of tubulin posttranslational modifications in conifer cells and reports that acetylated tubulin, but not detyrosinated tubulin was found in these cells. It also determined that some components of animal MTOCs are present in plant cells, but their localization did not always coincide with microtubule nucleating functions. These results combined with future studies on a wider range of plant cell types and plant genera will provide a greater understanding of the processes involved in plant microtubule dynamics.

6.4 References

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