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Tyrosinated, but not detyrosinated, α-tubulin is present in root tip cells

Rapid communication

S. Gilmer[1], **P. Clay**[1], **T. H. MacRae**[2], and **L. C. Fowke**[1,*]

[1] Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan and [2] Department of Biology, Dalhousie University, Halifax, Nova Scotia

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Summary. The distribution of tyrosinated and detyrosinated tubulin in microtubule arrays of pine and onion cells was investigated by immunofluorescence techniques. Staining of isolated cells and methacrylate sections of *Pinus radiata* and *Allium cepa* root tips indicated that all microtubule structures contained tyrosinated tubulin but not the posttranslationally modified detyrosinated tubulin. The detyrosinated tubulin epitope was, however, created in vitro by treating both sections and fixed whole cells with carboxypeptidase A.

Keywords: Posttranslational modification; Tubulin; Tyrosination; Conifer; Angiosperm.

Introduction

Posttranslational modifications contribute to the heterogeneity of tubulin and modify the surface of microtubules (MacRae 1997). Examples of these modifications include acetylation, detyrosination, phosphorylation, polyglutamylation, and polyglycylation. Two of these, acetylation and detyrosination, have been well documented in animal cells partly because antibodies which recognize microtubules containing the modified forms are available. However, there is only scant information on modified tubulin in plant cells. Acetylation has been discovered in meristematic conifer cells (Gilmer et al. 1999) but not in meristematic angiosperm cells (unpubl. results).

Detyrosination modifies the carboxy 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 making it detyrosinated tubulin. The reverse process, tyrosination, is performed by the enzyme tubulin tyrosine ligase (MacRae 1997). The same α-tubulin can be detyrosinated and then retyrosinated repeatedly. Because the carboxypeptidase binds to 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 depolymerized (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 detyrosination often contain both types of tubulin – detyrosinated and tyrosinated – along their length, although there can be microtubules greatly enriched in one form or the other (Sullivan 1988).

In vertebrate cells, detyrosinated α-tubulin chemically marks stable microtubule arrays. It is found in stable portions of axon microtubules (Baas and Black

* Correspondence and reprints: Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, SK S7N 5E2, Canada.

1990, Bulinski and Gundersen 1991), stable microtubules of fibroblasts, and stable microtubules in the first stages of differentiation (Bulinski and Gundersen 1991). Detyrosination may mediate interactions with intermediate filaments (Gurland and Gundersen 1995) or the Golgi complex (Skoufias et al. 1990, Thyberg and Moskalewski 1993).

Many studies have indicated that detyrosination is not directly responsible for any associated stability. In in vitro studies, microtubules enriched in detyrosinated and tyrosinated tubulin have similar instability parameters (Idriss et al. 1991) and polymerize equivalently (Raybin and Flavin 1977, Kumar and Flavin 1982).

Very few reports exist about the presence of detyrosinated tubulin in plant cells. Tyrosinated, but not detyrosinated, microtubules were detected in a diatom (Machell et al. 1995), in the spermatogenous cells of *Ceratopteris richardii* (Hoffman and Vaughn 1995a, b), and in pollen tubes of *Nicotiana tabacum* (Åström 1992, Del Casino et al. 1993). The presence of detyrosinated microtubules in pea stem cells was demonstrated indirectly by comparing staining of Western blots using antibodies for tyrosinated tubulin with staining using an antibody which recognizes both tyrosinated and detyrosinated microtubules (Duckett and Lloyd 1994). Another study demonstrated the presence of detyrosinated tubulin as well as other posttranslational modifications in fixed cells and immunoblots of cultured tobacco cells (Smertenko et al. 1997). Detyrosinated-tubulin staining was detected in all microtubule arrays in all stages of the cell cycle, but only in sporadic dotlike segments of these microtubules.

Because so little is known about the presence of detyrosinated tubulin in plant cells, this study was undertaken to determine if and where detyrosinated microtubules are located in plant cells. When plant cells were tested with antibodies raised against tyrosinated and detyrosinated tubulin, no evidence of the modified form in plant cells was found, whereas unmodified tyrosinated tubulin was present in all microtubule arrays. The detyrosinated tubulin epitope was, however, created in vitro by treating both sections and fixed whole cells with carboxypeptidase A.

Material and methods

Plant material

Pinus radiata seeds (a gift from A. Hardham, Australian National University) were surface sterilized in 30% hydrogen peroxide, rinsed in five 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- to 12-day-old seedlings were fixed as described below. *Allium cepa* (cv. Walla Walla) surface-sterilized seeds were germinated on filter paper over vermiculite. Root tips (3–6 mm) of 7- to 9-day old seedlings were fixed.

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: 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 2 mM $MgSO_4$, pH 6.9), for 1.5 h at room temperature, washed and dehydrated to 100% ethanol over 6 h, in increments of 10, 30, 50, 70, and 90% with three 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 to 4 parts butyl methacrylate, 0.5% benzoin ethyl ether, and 10 mM dithiothreitol with oxygen displaced by bubbling nitrogen through it for 1 h 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 h, and this was followed by two changes of 100% methacrylate over 24 h all at –20 °C. Each root tip was transferred to an individual modified BEEM embedding capsule (size 00; 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 h before exposure to UV light (10 cm from a lamp with a power of 20 W and a wavelength of 365 nm) 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 thick, 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 by shaking slides slowly for 10 min on a rotary shaker. The sections were moved to 100% ethanol and rehydrated in four steps to 30% ethanol in 0.85% sodium chloride (Kronenberger et al. 1993) before transfer to phosphate-buffered saline (PBS), pH 7.3, for 5 min and blocking with PBSB (1% protease-free BSA in PBS) for 20 min at room temperature.

Preparation of isolated root tip cells

Pinus radiata and *Allium cepa* root tips, 3–6 mm long, were prepared as described by Wick (1993). Samples were fixed for 1–1.5 h with 4% methanol-free formaldehyde (Polysciences) in MtSB containing 10% dimethyl sulfoxide and 0.1% Triton X-100, followed by three washes for 10 min each in MtSB. Fixed root tips were digested for 1 h (pine) or 30 min (onion) in 1% Cellulysin (Calbiochem) in MtSB with 0.3 mM phenylmethyl sulfonyl fluoride and 19 mM leupeptin, then washed three times 20 min in MtSB. 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% Triton X-100 in MtSB for 20 min and washed three times 1 min in PBS. Cells were preblocked with PBSB for 20 min at room temperature.

Immunofluorescence staining of methacrylate sections and isolated cells

The antibodies used were affinity-purified polyclonal antibodies or serum, anti-Y and anti-E that recognize tyrosinated microtubules and detyrosinated tubulin respectively (Xiang and MacRae 1995). Affinity-purified antibodies were applied full strength, the serum

diluted 1/500 or 1/1000 in PBSBT (PBSB with 0.5% Tween-20). No differences were observed between staining patterns between the affinity-purified antibodies and serum. A monoclonal antibody against chicken tubulin (Amersham N357) was diluted 1/250 in PBSBT, applied to the sections or cells and incubated in a humid chamber at 4 °C overnight and then at 37 °C for 1 h. Slides were washed with a stream of PBS, then in three changes of PBS over 1.5 h. Negative controls were exposed to PBSB in place of the primary antibodies. Sections or cells were incubated in secondary antibodies (goat anti-mouse Cy3 and/or goat anti-rabbit dichlorotriazinyl amino fluorescein [Jackson Immunoresearch] diluted 1/500 or 1/100 in PBSBT respectively) and incubated for 1 h at 37 °C in a moist chamber. Slides, washed as for the primary antibody and then in deionized, glass-distilled water (ddH_2O) for 5 min, were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (2.5 μg/ml) in ddH_2O for 2 min, before washing in ddH_2O and mounting in Fluorsave (Calbiochem). For fluorescence microscopy, slides were examined with a Zeiss Axioplan fluorescence microscope fitted with standard filters and a ×63 Apochromatic oil immersion objective lens. Ilford XP2-400 film was used for photography, and plates were assembled by Adobe Photoshop 5.0.

Treatment with carboxypeptidase

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. Intact cells were exposed to carboxypeptidase A (Sigma C9268), after the cells were fixed and extracted with Triton X-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 min at 37 °C, then washed with a stream of PBS, and then three changes of PBS over 20 min. Control sections or cells were treated with PBS in place of carboxypeptidase A.

Results

Distribution of tyrosinated and detyrosinated tubulin in methacrylate-embedded sections of plant tissue

All microtubule arrays in onion root tip cells were consistently stained with anti-Y, a polyclonal antibody that recognizes tyrosinated tubulin (α-tubulin that has retained the terminal Tyr residue) (Fig. 1a). In contrast, no recognizable microtubule structures were observed with anti-E, an antibody to detyrosinated tubulin (Fig. 1b), even though microtubule structures were highly visible with an antibody to β-tubulin (not shown). Sections treated with 0.05 units of carboxypeptidase A per ml, and subsequently stained with anti-E, the antibody to detyrosinated tubulin (Fig. 1c), exhibited a remarkable similarity to those undigested sections stained with the antibody to tyrosinated tubulin in that all microtubule arrays were stained. Figure 1b was a neighboring section in the same experiment treated in exactly the same way as the section shown in Fig. 1c except that it was exposed to MtSB instead of carboxypeptidase A. The same results were obtained with similarly treated *Pinus radiata* and *Pinus contorta* root tip sections (not shown).

Distribution of tyrosinated and detyrosinated tubulin in whole isolated cells

Supporting the results with sectioned tissues, tyrosinated tubulin was present in all microtubule arrays in whole isolated pine root tip cells (Fig. 2a). Typical β-tubulin staining showing intense staining of all microtubule arrays in another group of cells is presented (Fig. 2b) for comparison. In contrast, no microtubule structures had detectable levels of detyrosinated tubulin (e.g., Fig. 2c, d) even in those cells with abundant microtubules stained with the antibody to β-tubulin (Fig. 2d′). In cells treated with carboxypeptidase A, microtubules were detected by both anti-E, which recognizes detyrosinated tubulin (Fig. 2e, f), and the antibody to β-tubulin (Fig. 2e′, f′). Whole cells isolated from onion root tips also had no detectable detyrosinated tubulin except when treated with carboxypeptidase. After this treatment there was substantial staining with anti-E, the antibody which recognizes detyrosinated tubulin (not shown).

Discussion

This study indicates that detyrosinated tubulin is not present at detectable levels in two species of pine and onion root tip cells. Furthermore, we provide evidence that the microtubules in these cells have abundant tyrosinated tubulin, indicating that this unmodified form is the major variant in these cells. The fact that detyrosinated tubulin is 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 a failure of the antibodies to recognize detyrosinated α-tubulin in plant cells. This is the first report of the creation in plant sections or cells of the epitope recognized by antibodies to detyrosinated tubulin with carboxypeptidase A, an enzyme isolated from bovine pancreas. The creation of the detyrosinated epitope on nitrocellulose blots has been reported (Xiang and MacRae 1995).

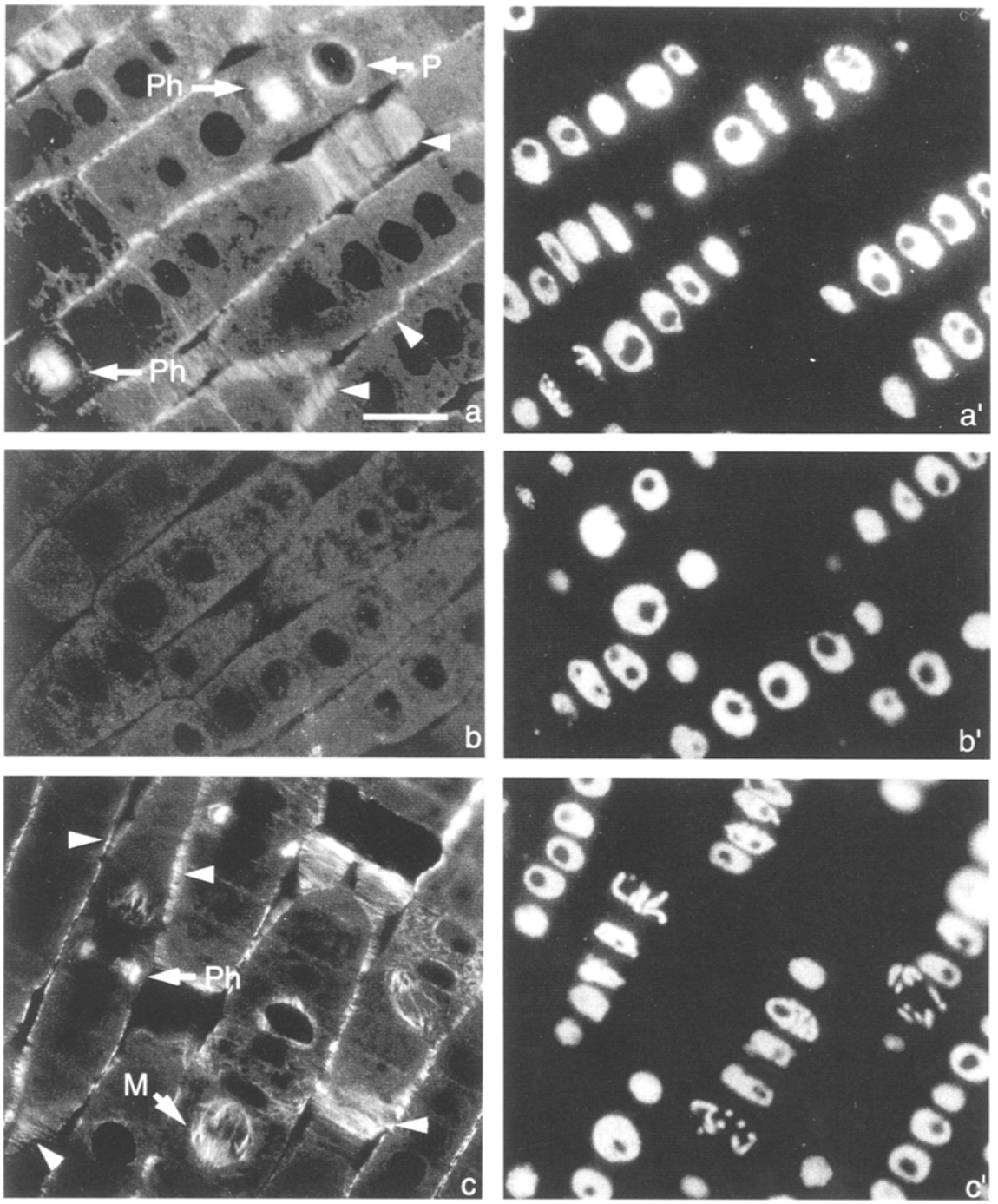

Fig. 1a–c. Methacrylate sections of *A. cepa* root tips. All micrographs are at the same magnification, bar: 20 μm. **a** All microtubule structures stained with anti-Y (which recognizes tyrosinated tubulin) in this oblique onion root tip section. Note prominent phragmoplasts (*Ph*), prophase spindle (*P*), and cortical microtubules (arrowheads). **a′** DAPI staining of the same section. **b** No microtubules detected with anti-E (which recognizes detyrosinated tubulin). **b′** DAPI staining of the same section. **c** All microtubule arrays stained with anti-E (which recognizes detyrosinated tubulin) after carboxypeptidase A treatment, including a phragmoplast (*Ph*), metaphase spindle (*M*), and cortical microtubules (arrowheads). **c′** DAPI staining of the same section

Our report of the presence of tyrosinated tubulin in all microtubule arrays confirms 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 thaliana* code for a tyrosinated form (Kopczak et al. 1992). Our results differ from two recent reports indicating the presence

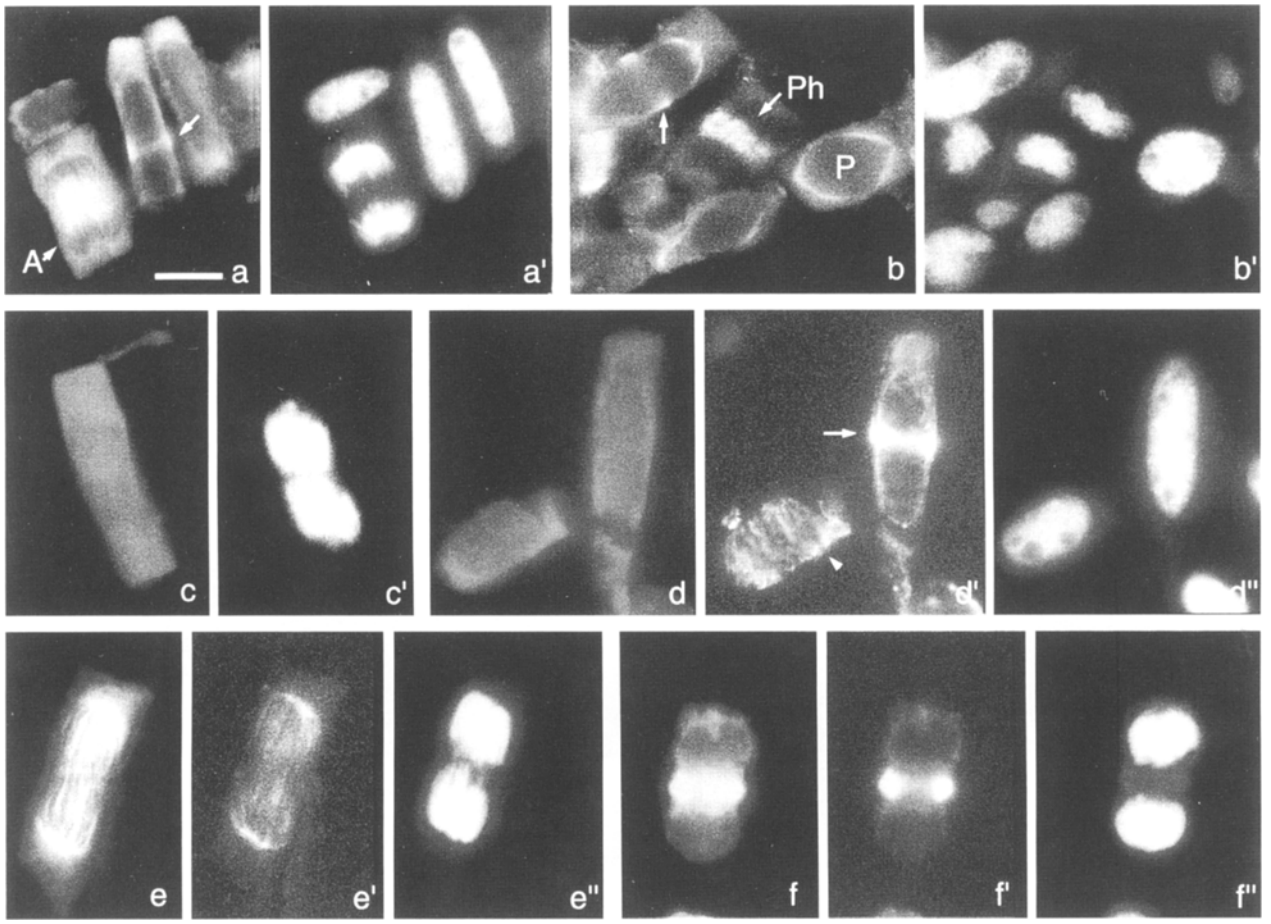

Fig. 2 a–f. Whole isolated *P. radiata* root tip cells. All micrographs are at the same magnification, bar: 10 μm. **a** Interzonal spindle microtubules in a cell in late anaphase (*A*) and a preprophase band (arrow) are both stained with anti-Y (which recognizes tyrosinated tubulin). **a′** DAPI staining of the same cells. **b** All microtubule arrays stained with an antibody to β-tubulin including phragmoplast (*Ph*), prophase spindle (*P*), and preprophase band (arrow). **b′** DAPI staining of nuclei. **c** No staining of spindle microtubules with anti-E (which recognizes detyrosinated tubulin). **c′** DAPI staining of nuclei. **d** No staining of microtubules with anti-E. **d′** Same cells with cortical microtubules (arrowhead) and preprophase band (arrow) stained with an antibody to β-tubulin. **d″** DAPI staining of nuclei. **e** Spindle microtubules stained with anti-E after the cell was treated with carboxypeptidase A. **e′** Same cell stained with β-tubulin. **e″** Chromosomes stained with DAPI. **f** Phragmoplast stained with anti-E after the cell was treated with carboxypeptidase A. **f′** Same cell stained with β-tubulin. **f″** Reforming nuclei stained with DAPI

of detyrosinated tubulin in angiosperm cells (Duckett and Lloyd 1994, Smertenko et al. 1997). Although this could be due to differences in sample preparation or antibody sensitivity, that is not very likely in light of the fact that detyrosinated tubulin was detected in our material when the epitope was artificially created. The fact that detyrosinated tubulin was found in Nicotiana cultured cells (Smertenko et al. 1997) 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 in their immunofluorescence results the modified forms of tubulin were found in tobacco cultured cells in very small quantities. In our studies, such small quantities may have gone undetected. However, we would not have expected to find very small quantities of detyrosinated tubulin given the results from animal studies that this modified form is present in abundance when it is present.

The evidence for detyrosinated tubulin in elongating internodal regions of gibberellic acid-treated dwarf pea plants (Duckett and Lloyd 1994) could be the result of selective stabilization associated with the effects of gibberellic acid exposure. However, this is puzzling because it would indicate that the detyrosination is very different from that which occurs in animal cells. This plant detyrosination seems to be

divorced from the detyrosination-retyrosination cycle. In animal cells, tubulin dimers are retyrosinated shortly after detyrosinated tubulin is depolymerized. 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). If these plant cells have tubulin tyrosine ligase that functions as it does in animal cells, then there should be tyrosinated tubulin in the elongating plant internodes. However, no tyrosinated version of α_1- and little tyrosinated α_2-tubulin was detected in this study (Duckett and Lloyd 1994).

In animal cells detyrosination is associated with (1) highly stable microtubules such as those in nerve cells which may have a half-life of 2.2 h (Laferrière et al. 1997), (2) a specialized set of microtubules that is stabilized in the early stages of animal cellular differentiation (Bulinski and Gundersen 1991), or (3) microtubules associated with the Golgi apparatus (Skoufias et al. 1990, Thyberg and Moskalewski 1993) or intermediate filaments (Gurland and Gundersen 1995). All of these conditions are specific to animal cells. Nerve cells have highly specialized cytoskeletons and their own probably overlapping and redundant system of controls which regulate their dynamics. They likely have little similarity with processes in plant cells. Cellular morphogenesis is very different in plants and animals. The structure of the Golgi apparatus and probably the distribution of intermediate filaments are also dissimilar in plants and animals. In light of these major 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. Both earlier reports of detyrosination in plant cells are consistent with the possibility that the plant tubulin is modified by another carboxypeptidase present in these plant cells and that a tubulin tyrosine ligase and a specific tubulin carboxypeptidase, present in animal cells, are not present in plant cells.

Further studies focussing on different tissues, and a broader survey of plant species, are needed to determine the extent of detyrosination in plant cell microtubules. The isolation of a plant tubulin carboxypeptidase and tubulin tyrosine ligase would indicate that detyrosination is similar in plants and animals. Without this, it is possible that the detyrosination of plant tubulin is a nonfunctioning side effect of another cellular carboxypeptidase. Detyrosination may very well occur by 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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