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A microtubule-associated protein in maize is expressed during phytochrome-induced cell elongation

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Summary

Plants can adapt their shape to environmental stimuli. This response is mediated by the reorganization of cortical microtubules, a unique element of the cytoskeleton. However, the molecular base of this response has remained obscure so far. In an attempt to solve this problem, signal-dependent changes in the pattern of microtubule-binding proteins were analysed during coleoptile elongation in maize, that is, under the control of the plant photoreceptor phytochrome. Two putative MAPs of 100 kDa (P_{100}) and 50 kDa apparent molecular weights were identified in cytosolic extracts from non-elongating and elongating cells. Both proteins co-assembled with endogenous tubulin, bound to neurotubules and were immunologically related to the neural MAP τ: the P_{100} protein, depending on the physiological situation, was manifest as a double band and was always found to be heat-stable. In contrast, the 50 kDa MAP was heat-stable only for particular tissues and physiological treatments. The P_{100} protein was present in all tissues, however in a reduced amount in elongating coleoptiles. The 50 kDa MAP was expressed exclusively upon induction of phytochrome-dependent cell elongation. As shown by immunofluorescence double-staining, an epitope shared by both proteins colocalized with cortical microtubules *in situ*, but exclusively in elongating cells. In non-elongating cells, only the nuclei were stained. Partially purified nuclei from elongating cells were enriched in P_{100}, whereas the 50 kDa MAP became enriched in a partially purified plasma membrane fraction.

Introduction

Plants can adapt their growth and development to signals, they perceive from the environment. This requires the control of cellular morphogenesis by signal-transduction chains. This control is a unique aspect of plant morphogenesis (Nick and Furuya, 1992). It appears that cortical microtubules play a key role in this process: they can control cellular shape by defining the direction of cellulose microfibrils and thus the mechanical properties of the cell wall (Giddings and Staehelin, 1991; Green, 1980). Moreover, they respond readily by reorientation to signals such as gravity (Blancaflor and Hasenstein, 1993), red light (Zandomeni and Schopfer, 1993), blue light (Nick *et al.*, 1990), and plant hormones (Shibaoka, 1991).

The spatiotemporal organization of the microtubule network in cells of animals and lower plants is based upon the microtubule-nucleating activity of the centrosomes (Cande, 1990). Higher plants, however, lack such centrosomes. The activity of their functional homologues, the microtubule-organizing centres (MTOC), is thought to be subject to spatial and temporal control (Lambert, 1993). In some cells, the nuclear envelope was shown to embody a MTOC-activity (Stoppin *et al.*, 1994; Vantard *et al.*, 1990; Zhang *et al.*, 1990). In addition, microtubule-nucleating sites adjacent to the plasma membrane have been reported, that might be important for the control of cell shape (Falconer *et al.*, 1988; Hasezawa and Nagata, 1993; Marc and Palevitz, 1990). These nucleating activities of MTOC are thought to be accompanied by considerable intracellular translocation of polymerized microtubules. Both processes seem to contribute to the spatial order of the microtubule cytoskeleton.

At the biochemical level, the dynamics of the microtubule cytoskeleton can be controlled by the activity of specific microtubule-associated proteins (MAP). This group of proteins was originally defined by two criteria: first, they fractionate with microtubules during assembly and disassembly, and, second, they are able to stimulate microtubule nucleation and elongation from purified tubulin *in vitro* (Kreis and Vale, 1993). This definition of microtubule-associated proteins has later been questioned, since nucleation and elongation are likely to be distinct processes *in vivo*. It has been suggested instead that the attachment to microtubules *in vivo* be chosen as the decisive criterion for a microtubule-associated protein (Solomon *et al.*, 1979). Among plant biologists, too, the term MAP has been under debate recently (Durso and Cyr, 1994; Morejohn, 1994). In this paper, the term MAP will be used for proteins fulfilling the criterion of attachment to microtubules *in vivo*. Although an extensive body of information has been accumulated on such proteins from animals and especially neural cells (Kreis and Vale, 1993), little is known about MAPs in plants. This is illustrated by the fact that, so far, no plant MAP has been cloned and sequenced.

Recently, a heat-stable 100 kDa protein has been purified from a maize suspension culture that exhibited several characteristics of a MAP, such as binding to taxol-stabilized

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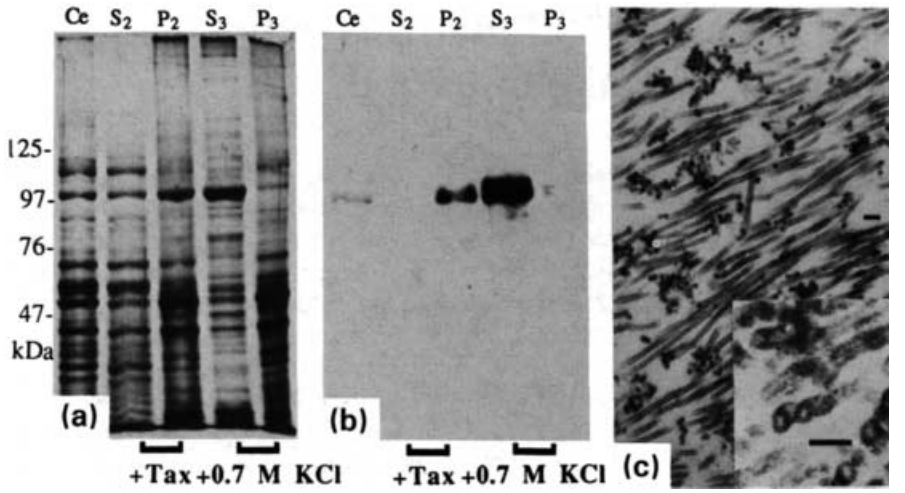

Figure 1. Assay for co-assembly with endogenous tubulin in extracts from non-elongating cells.
(a) Silver stain according to Ansorge (1982), (b) Western blot probed with anti-P_{100} antibodies. Cytosolic extracts (Ce) were incubated at 27°C with 20 μM taxol in the presence of 0.15 M KCl. Proteins, capable of co-assembly with endogenous tubulin were collected by centrifugation in the pellet (P_2), whereas unbound proteins remained in the supernatant (S_2). The pellet was resuspended in 0.7 M KCl and, after a second centrifugation step, putative MAPs remained in the supernatant (S_3), defined as the MAP-fraction, whereas the endogenous microtubules were collected in the pellet (P_3). Two micrograms of total protein were loaded per lane for the silver stain, 10 μg per lane for the Western blot.
(c) Electron microscopical image of a microtubule pellet. Black circles correspond to microtubules that were in an orthogonal orientation (see inset). Bars indicate 0.1 μm.

neurotubules and co-polymerization *in vitro* with purified neural tubulin (Vantard *et al.*, 1994). This protein (P_{100}) is found to be widely distributed in plant-cell cultures.

To get some insight into the function of the P_{100} protein, a developmental analysis of its expression was initiated in maize seedlings focusing upon the coleoptile. The Graminean coleoptile, in sharp contrast to the suspension cell cultures used previously, grows exclusively by cell expansion, not by cell division (Rothert, 1894). Moreover, its growth responds to various signals such as light (Mohr, 1972), gravity (Pickard, 1985), and auxin (Briggs, 1963a), and these responses are accompanied by corresponding reorientations of cortical microtubules (Nick *et al.*, 1990; Zandomeni and Schopfer, 1993). Dark-grown etiolated coleoptiles of maize barely elongate, whereas elongation is stimulated up to fourfold after irradiation with light of the red part of the spectrum (Briggs, 1963b), mediated by the plant photoreceptor phytochrome (Furuya, 1993). The situation in non-cycling, non-elongating cells (etiolated coleoptiles) was compared with the situation in non-cycling, but elongating, cells (coleoptiles with an induced phytochrome system) in an attempt to identify plant MAPs related to cell elongation.

Results

Is the expression of microtubule-binding proteins correlated to cell elongation in maize?

Cytosolic extracts from non-elongating cells of coleoptiles cultivated in complete darkness were compared with cytosolic extracts from cells, where elongation had been stimulated by induction of the phytochrome system with respect to the presence of microtubule-binding proteins.

First, the extracts were assayed for proteins, capable of co-assembly with endogenous microtubules (Figures 1 and

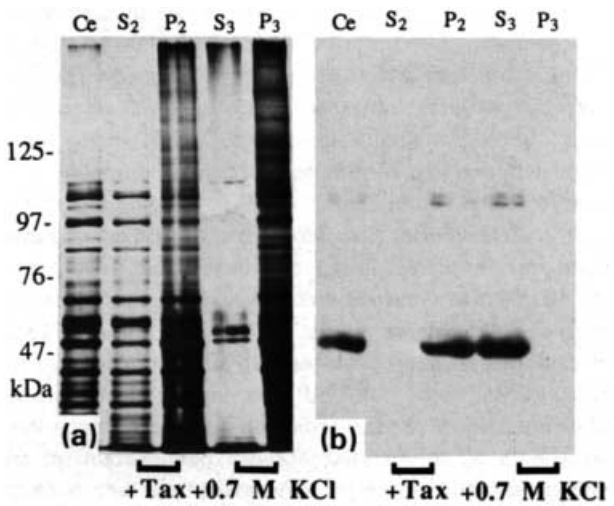

Figure 2. Assay for co-assembly with endogenous tubulin in extracts from elongating cells.
For details refer to Figure 1.

2). Since the concentration of endogenous tubulin in the cytosolic extract did not suffice for spontaneous formation of microtubules, 20 μM taxol had to be added and the resulting microtubules were sedimented in the presence of low (0.15 M) concentrations of KCl (fraction P_2). Then, putative microtubule-binding proteins were detached by washing the pellet with high (0.7 M) concentrations of KCl, whereas the pellet (fraction P_3) consisted of microtubules, that is, tubulin and products of tubulin degradation (Figure 1c). The resulting supernatant S_3 is referred to as a MAP-fraction. The protein patterns of the MAP-fraction were different for extracts obtained from non-elongating cells compared with extracts from elongating cells. In extracts from non-elongating cells (Figure 1a), a band of about 100 kDa dominated. In contrast, in the MAP-fraction

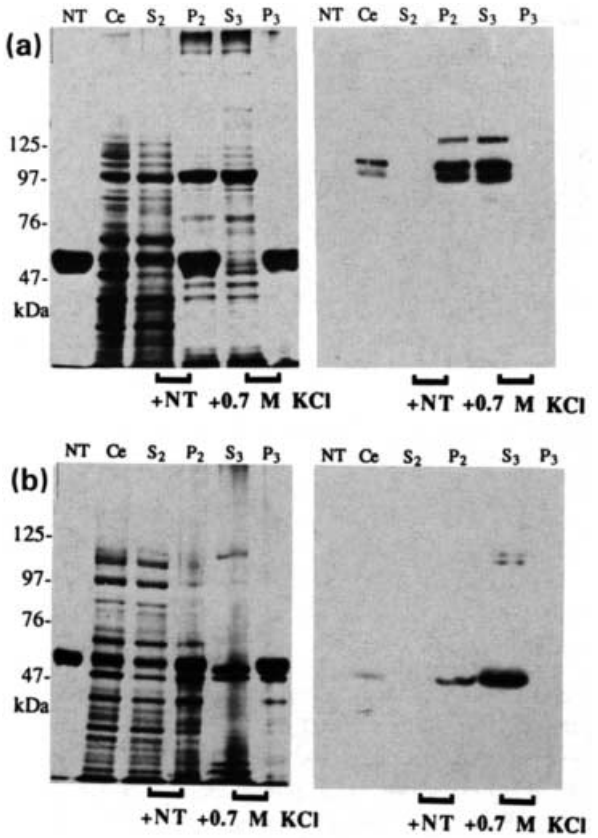

Figure 3. Neurotubule-co-sedimentation assay.
Western blots probed with anti-P_{100} antibodies. Neurotubule-binding proteins accumulate in the MAP-fraction S_3. The assay was performed with extracts from non-elongating, etiolated coleoptiles (a) and with extracts from light-treated elongating coleoptiles (b). Two micrograms of total protein were loaded per lane for the silver stain (left), 10 μg per lane for the Western blot (right).

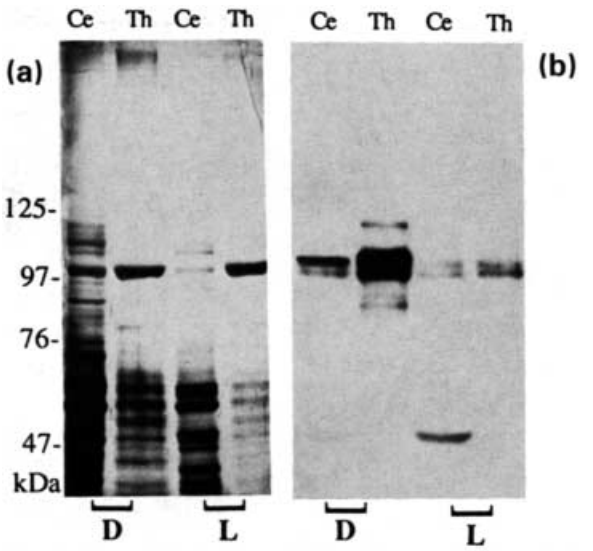

Figure 4. Assay for heat-stability in extracts from elongating and non-elongating coleoptile cells.
(a) Silver stain according to Ansorge (1982), (b) Western blot probed with anti-P_{100} antibodies. Cytosolic extracts (Ce) were boiled in the presence of 0.75 M NaCl. Thermostable proteins (Th) remained soluble under these conditions. Extracts obtained from non-elongating, dark-grown cells (D) were compared with extracts from cells, where elongation had been induced by light (L). Two micrograms of total protein were loaded per lane for the silver stain, 10 μg per lane for the Western blot.

from elongating cells (Figure 2a) this 100 kDa protein was much less abundant, whereas another protein of about 50 kDa appeared. Both, the 100 kDa and the 50 kDa protein (Figures 1b and 2b) were recognized on immunoblots by the antibody raised against the P_{100} protein from maize cell cultures.

Since the P_{100} from maize cell cultures is immunologically related to the neural MAP τ, it was interesting to test whether the P_{100} protein and the 50 kDa protein were capable of binding to neurotubules. The result of this assay was positive: in the corresponding MAP-fractions (Figure 3), the P_{100} protein was dominant, if neurotubules were incubated with extracts from non-elongating cells (Figure 3a), whereas for elongating cells the amount of this protein was diminished and the 50 kDa protein prevailed. In addition, the antibody against the P_{100} protein detected an accompanying band of 125 kDa in the MAP-fraction from non-elongating cells (Figure 3a).

The results from those experiments demonstrate that:

(i) the P_{100} putative MAP from maize cell cultures (Vantard *et al.*, 1994) is also expressed in differentiated cells;
(ii) the expression, and/or the capacity of this protein to bind microtubules, is regulated during development;
(iii) an immunologically related putative MAP of 50 kDa is expressed in differentiated, elongating cells;
(iv) both putative plant MAPs bind to neurotubules.

Properties of the putative 50 kDa MAP from maize

The P_{100} protein from maize cell cultures (Vantard *et al.*, 1994) has been found to be heat-stable. Since the putative 50 kDa MAP from elongating cells is immunologically related to the P_{100} protein, it was worth testing, whether it is heat-stable as well. Moreover, this property may be useful for the purification of this protein. In extracts from non-elongating coleoptiles, the P_{100} protein was found to be heat-stable, as well as the accompanying 125 kDa protein (Figure 4b, D). The same result was principally obtained for extracts from elongating cells (Figure 4b, L), although the 100 kDa band was much weaker as compared with the extracts from dark-grown cells. In contrast, the putative 50 kDa MAP, found in cytosolic extracts from elongating coleoptiles, was not heat-stable (Figure 4b, L). As a comparative control, thermostable extracts from dark-grown and illuminated roots were analysed (Figure 5a). In both types of extracts, the P_{100} protein was found to be heat-stable. It should be mentioned that this protein, in

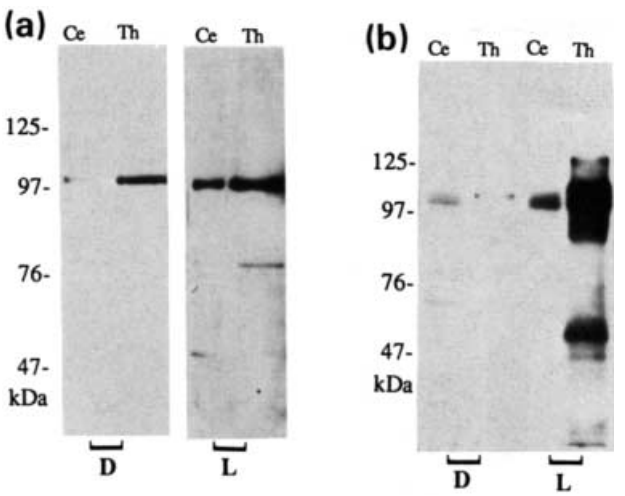

Figure 5. Assay for heat-stability in extracts from roots and mesocotyls. Extracts from dark-grown (D) and light-treated (L) roots (a) and mesocotyls (b) were probed with anti-P_{100} antibodies. For details refer to Figure 5.

contrast to coleoptiles, never turned up as a double band in the root (Figure 5a). Only a barely perceptible trace of the 50 kDa protein could be detected by the antibody in extracts of light-treated roots, in contrast to the situation in light-treated coleoptiles (Figures 4 and 5a). The roots did respond to the light, however, as was seen from changes in gravitropism, formation of root hairs, thickness, and length (data not shown). Moreover, a thermostable protein of 83 kDa was detected by the anti-P_{100} antibody in light-treated roots, but not in dark-grown roots (Figure 5a, L). A similar picture was obtained, when this type of experiment was performed with the mesocotyl (the stalk of the seedling below the first node). Again, the P_{100} protein was recognized by the antibody, although the strength of the signal was weaker than for the coleoptile (Figure 5b). Surprisingly, for light-treated mesocotyls, the 50 kDa protein was found to be heat-stable.

The P_{100} protein and the 50 kDa protein were partially purified from maize mesocotyls by thermostable extraction followed by anion-exchange chromatography. The P_{100} protein was prepared from dark-grown, etiolated mesocotyls (Figure 5b), whereas small amounts of the 50 kDa protein could be obtained from light-treated mesocotyls (Figure 6b). Maize tubulin from cytosolic extracts of etiolated mesocotyls was purified by anion-exchange chromatography and used as a control. The three proteins were probed with various antibodies. Antibodies directed against β-tubulin did not recognize either the P_{100} protein (Figure 6a, T) or the 50 kDa protein (Figure 6c, lane 2), and the same was found for antibodies against α-tubulin (data not shown). Moreover, the antibody against the heat-stable P_{100} protein did not react with the endogenous tubulin (Figure 6a, H). However, a specific response to both proteins (Figure 6a, H for the P_{100} protein; Figure 6c, lane 3 for the 50 kDa protein) was obtained with an affinity-purified antibody directed against the neural MAP τ (Vantard *et al.*, 1991).

This first characterization of protein properties shows that:

(i) the P_{100} protein is heat-stable in all tissues tested so far, as found previously in maize cell cultures (Vantard *et al.*, 1994);
(ii) the P_{100} protein is present as a single band in root and mesocotyl tissue, as found previously in maize cell cultures, but is expressed as a double band in coleoptiles;
(iii) the putative 50 kDa MAP, with one exception (light-treated mesocotyls) is not thermostable;
(iv) both proteins are immunologically related to the neural MAP τ.

The 50 kDa MAP co-localizes with cortical microtubules in situ

The criterion for a MAP is the co-localization with microtubules *in vivo* (Solomon *et al.*, 1979). Therefore, the localization of the two epitopes recognized by the anti-P_{100} antibody was assayed by double-immunofluorescence staining in maize coleoptiles. Coleoptile elongation was followed over time for a light treatment saturating the induction of the phytochrome system (Figure 7a). After a lag phase of approximately 1–1.5 days after sowing, rapid length growth lasting to 3 days after sowing was observed and, within 4 days, a final length of about 40 mm was reached. In contrast, dark-grown coleoptiles do not grow longer than about 10 mm during that period (Figure 7a). Cytosolic extracts were obtained at various time points during light-induced coleoptile elongation and probed with the P_{100} antibody. For mature embryos and up to 1 day after sowing (Figure 7b), only the 100 kDa double-band known from dark-grown coleoptiles (Figure 1b) was detected by this antibody. During the transition to rapid coleoptile elongation (between 1 and 2 days after sowing), the lower of the two 100 kDa bands disappeared, and the 50 kDa protein appeared. It should be mentioned that, simultaneously, the upper of the two 100 kDa bands seemed to be slightly shifted to a higher apparent molecular weight and maintained a strong expression.

The epitopes recognized by this antibody were probed by immunofluorescence at various stages of coleoptile elongation (Figure 8). Then, the relation of these epitopes with microtubules was assayed using a double-stain in epidermal cells, the main target for growth control in coleoptiles (Kutschera, 1991). In mature embryos, where exclusively the P_{100} protein is expressed (Figure 7b), no co-localization of the immunoreactive epitope with microtubular structures could be detected. Instead, a clear staining of nuclei (as

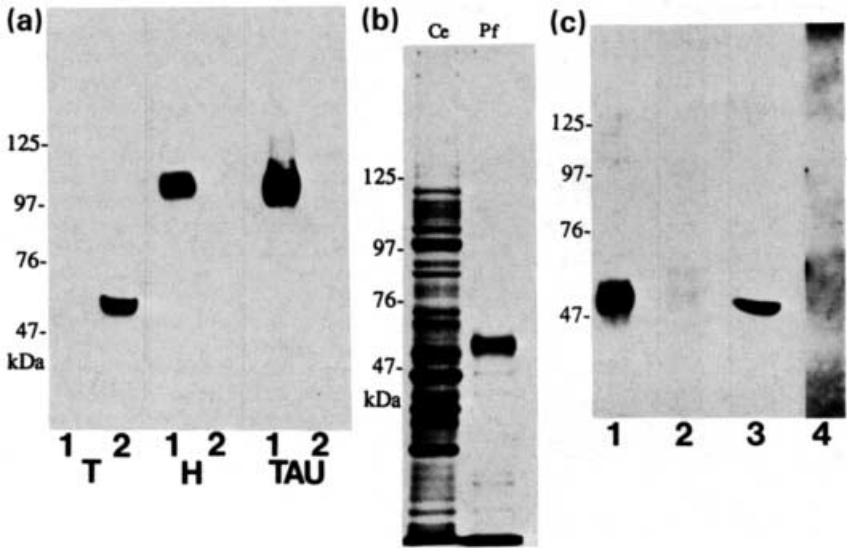

Figure 6. Immunological relationship between microtubule-binding proteins.
(a) Immunological comparison of P_{100} protein purified from dark-grown mesocotyls (lane 1) with tubulin from the same tissue (lane 2). Ten micrograms of total protein per lane were probed with antibodies directed against β-tubulin (T), anti-P_{100} antibodies (H), and antibodies against neural τ (TAU).
(b) Partial purification of the putative 50 kDa MAP from mesocotyls treated by constant far-red light. Ce cytosolic extract, Pf purified fraction. Two micrograms of total protein loaded per lane.
(c) Immunological characterization of the putative 50 kDa MAP. Western blots with 2 μg of the purified fraction per lane were probed with anti-P_{100} antibodies (lane 1), antibodies directed against β-tubulin (lane 2), antibodies directed against neural τ (lane 3), and, as a negative control, the secondary antibody alone (lane 4)

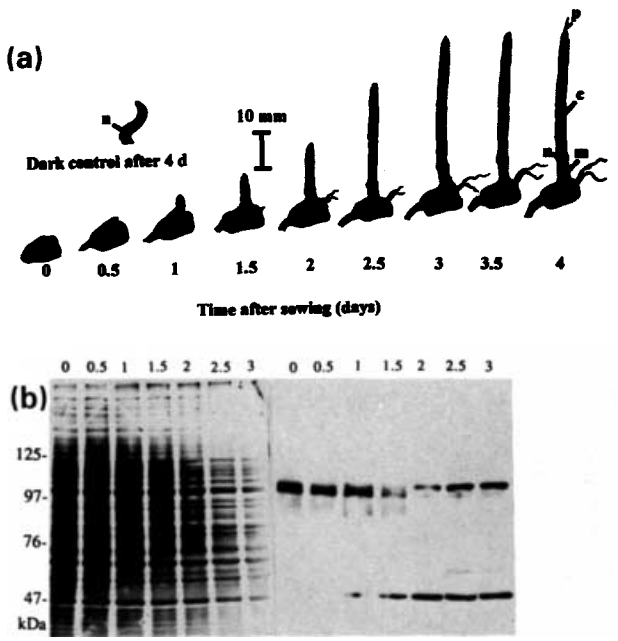

Figure 7. Expression of putative MAPs during light-induced coleoptile elongation.
(a) Coleoptile elongation in continuous far-red light: p, primary leaf; n, first node separating coleoptile c and mesocotyl m. Note the short and curved coleoptiles after 4 days of cultivation in complete darkness.
(b) Detection of the 100 kDa and the 50 kDa microtubule-binding proteins in cytosolic extracts obtained from elongating coleoptiles after various time intervals of cultivation in continuous far-red light. Left: silver stain according to Ansorge (1982), right: Western blot probed with antibodies raised against the heat-stable 100 kDa protein from maize suspension cultures (Vantard et al., 1994). Ten micrograms of total protein were loaded per lane.

identified by DAPI-staining, data not shown) was observed (Figure 8a). The specificity of this label was verified by controls, where the primary antibody was omitted or replaced by the respective pre-immune serum. No staining of nuclei was observed in these control experiments (data not shown). In contrast, a clear co-localization of the epitopes recognized by this antibody with cortical microtubules could be demonstrated during coleoptile elongation, when the 50 kDa microtubule-binding protein appears. Staining was very strong from 2.5 days after sowing (Figure 8c, d and e), but became detectable as early as 1.5 days after sowing (data not shown). It should be mentioned, however, that nuclei were stained in a manner similar to that observed in mature embryos. Again, the control experiment using the respective pre-immune serum in place of the antibody (Figure 8b), did not yield any signal that was co-localized with microtubules. However, unspecific staining of amyloplasts was observed in this negative control—in contrast to the nuclei stained under addition of the primary antibody these structures did not exhibit any DAPI-staining (data not shown).

The cross-reactivity of the antibody with both the P_{100} protein and the putative 50 kDa MAP hampers the unequivocal correlation of the immunofluorescence data with the data from the Western blot experiments. It is not clear, for instance, whether the putative 50 kDa MAP or the slightly upshifted upper P_{100} protein represents the epitope attached to cortical microtubules in the immunofluorescence double-label experiments. Therefore, a coarse subcellular fractionation was performed with tissue, where cell elongation had been stimulated by induction of the phytochrome system, and where both the P_{100} protein and the 50 kDa microtubule-binding protein were present. These experiments (Figure 9) showed that the P_{100} protein became enriched in the nuclear fraction, whereas the 50 kDa protein (as some of the tubulin) was partitioned to a purified plasma membrane fraction. The 50 kDa protein could not be solubilized by washing the membranes with Triton X-100, excluding a mere trapping of

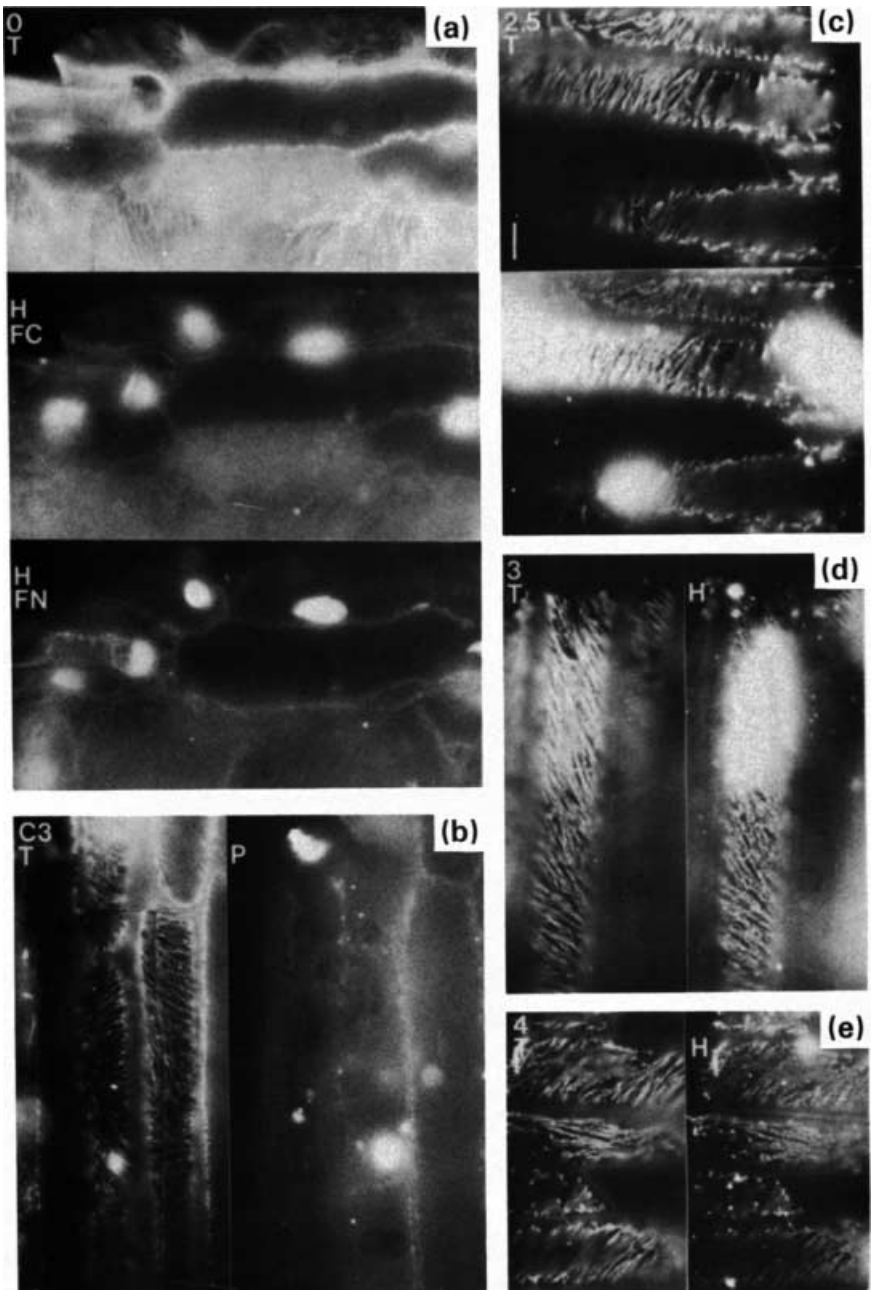

Figure 8. *In situ* detection of immunoreactive epitopes by immunofluorescence double labelling after 0 (a), 2.5 (c), 3 (d), and 4 (e) days of cultivation in continuous far-red light. T cortical microtubules stained by a mouse anti-β-tubulin antibody detected with a secondary antibody conjugated to fluorescein-isothiocyanate, H staining of the same cells with rabbit anti-P_{100} antibodies detected with a secondary antibody conjugated to rhodamin. FC focus on cortical microtubules, FN focus on the nuclei. The white bar represents 10 μm.
(b) Control experiment (C3) at 3 days after cultivation in continuous far-red light with replacement of the rabbit antibody against the heat-stable 100 kDa protein by the respective preimmune serum P. The fluorescent globules in (b) are amyloplasts that are unspecifically stained even by the fluorescent secondary antibody alone.

the protein in microsomal vesicles. This supports the view that the P_{100} protein is responsible for the immunofluorescence label of nuclei (Figure 8a), whereas it is the 50 kDa microtubule-binding protein that causes the label of cortical microtubules in elongating cells.

Discussion

The P_{100} protein is expressed in differentiated tissues

The cytoskeleton of higher plants is principally distinct from the cytoskeleton typical for animal cells: it lacks centrosomes and it includes a specialized population of microtubules adjacent to the cell wall, the cortical microtubules (Cyr, 1994; Lambert, 1993). Despite these unique features of the plant cytoskeleton, very little is known about the proteins guiding and regulating the spatial and temporal dynamics of microtubules in higher plants.

Nevertheless, various plant proteins have been isolated that are capable of binding to neurotubules. One of these proteins, the P_{100} protein, was first isolated from a maize suspension culture and was found to be widely distributed in cell cultures from various species (Vantard *et al.*, 1994). To obtain some insight into the possible physiological role

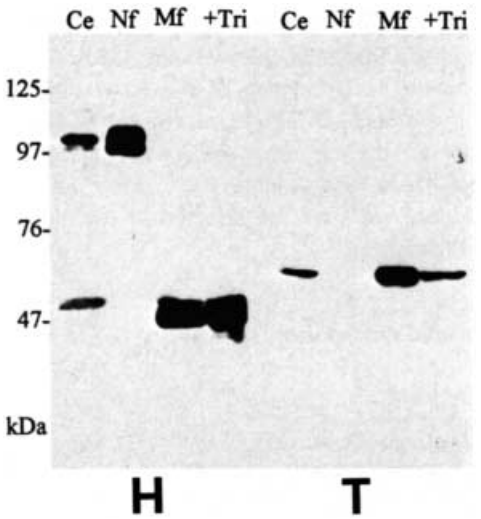

Figure 9. Subcellular fractionation of extracts from elongating cells. Cytosolic extracts (Ce), a partially purified nuclear fraction (Nf), a partially purified plasma-membrane fraction (Mf), and the same fraction washed with 0.5 % Triton X100 to account for unspecific trapping of soluble proteins (Tri) were probed with the antibody raised against the P_{100} protein (H) or β-tubulin (T). Ten micrograms of total protein were loaded per lane.

of this protein, various differentiated tissues were probed with the anti-P_{100} antibody.

The P_{100} was present in all differentiated cells tested so far, in etiolated as well as in light treated tissues (Figures 4 and 5a and b). It was able to co-assemble with endogenous microtubules (Figures 1 and 2). Moreover, it bound to neurotubules (Figure 3), was heat-stable (Figures 4 and 5a and b), and was found to be immunologically related to the neural MAP τ (Figure 6a). This shows that the P_{100} protein, in differentiated cells as well as in cultured cells (Vantard *et al.*, 1994) behaves as expected for a plant MAP.

Despite this apparently ubiquitous expression of the P_{100} protein in differentiated maize tissues, there seems to be a tissue-dependent developmental regulation: the amount of P_{100} is reduced upon phytochrome-induced cell elongation (Figure 4 and 7a and b). Moreover, whereas the P_{100} protein is present as a single band in extracts from roots and mesocotyls (Figures 5a,b), it clearly forms a double band in extracts obtained from coleoptiles (Figures 1–4). Thus, the situation in roots and mesocotyls resembles that in cultured cells (Vantard *et al.*, 1994).

The double band of P_{100}, observed in coleoptile extracts (Figures 1–4), might be either caused by expression of different isotypes of this protein, by post-translational modifications, such as phosphorylation, or by differential splicing. Although at this point one can only speculate about the real cause, it should be mentioned that all three mechanisms have been described for the neural MAP τ and MAP2 (Lee, 1993; Matus, 1990; Wiche *et al.*, 1991).

A 50 kDa MAP is expressed in elongating cells

In elongating cells, in addition to the P_{100} protein, a second putative MAP of 50 kDa was expressed. Like the P_{100} protein, it was able to co-assemble with endogenous microtubules (Figure 2), to bind to neurotubules (Figure 3), and, as the P_{100} protein, it was immunologically related to the neural MAP τ (Figure 6b and c). Moreover, it was recognized by the anti-P_{100} antibody (Figure 6c). However, in contrast to the P_{100} protein, it was found to be sensitive against heat (Figures 4 and 5a) with the only exception being light-treated mesocotyls (Figure 5b). Furthermore, preliminary results from peptide mapping (data not shown) suggest that the P_{100} protein belongs to the heat-shock protein 90 family of chaperones, whereas the peptide fragments obtained from the 50 kDa MAP did not exhibit any sequence similarity to published genes or proteins. Therefore, the 50 kDa protein is likely to be a new protein, distinct from the P_{100} protein, and not just a proteolytic fragment of the P_{100} protein. In order to address this problem more directly, it is planned to clone the corresponding gene(s).

The 50 kDa protein was expressed concomitantly with the onset of cell elongation (Figure 7a and b) and could not be detected in dark-grown coleoptiles, where elongation was blocked (Figures 2,3 and 4) or in coleoptiles that were still too young to respond to the elongation-inducing light signal (Figures 7a and b). In this respect, it should be mentioned that the 50 kDa putative MAP can be detected in etiolated coleoptiles of rice (data not shown), where cell elongation can proceed only in darkness (Pjon and Furuya, 1967). This suggests that, in Graminean coleoptiles, expression of the 50 kDa putative MAP is a marker for cell elongation.

The criterion for a MAP is co-localization with microtubules *in vivo* (Solomon *et al.*, 1979). This was tested for the 50 kDa putative MAP by double immunofluorescence labelling in epidermal cells of maize coleoptile, where the growth of this organ is controlled (Kutschera, 1991).

The epitope common to both proteins co-localized with cortical microtubules, but only if the 50 kDa protein was present (Figures 7b and 8). This suggests that the 50 kDa and not the P_{100} protein is attached to cortical microtubules.

However, simultaneously, the upper 100 kDa band is slightly shifted to higher apparent molecular weight and the underlying modification (for instance, a phosphorylation) might mediate the binding of this modified P_{100} protein to cortical microtubules. No antibody specific to the 50 kDa protein without cross-reactions has been available so far. Nevertheless, the finding that, in elongating cells, the 50 kDa protein and not the P_{100} protein becomes enriched in partially purified plasma membranes, together with a certain proportion of tubulin (Figure 9), is not in favour of this alternative interpretation. Moreover, the P_{100} proteins are found in nuclear fractions of elongating cells and a

nuclear localization of the corresponding epitope is observed in non-elongating, dark-grown tissue, where the 50 kDa protein is not expressed (Figure 8a).

Thus, the 50 kDa protein, according to the definition of Solomon *et al.* (1979), seems to be, indeed, a MAP, because it is co-localized with microtubules *in vivo* (Morejohn, 1994). Its association with cortical microtubules, a cytoskeletal element specific for plant cells, makes this MAP particularly interesting. The observation that the expression of the 50 kDa MAP is confined to elongating coleoptiles, suggests that this MAP might be related to the dramatic restructuring of the microtubule cytoskeleton accompanying the onset of cell elongation in this tissue (Nick *et al.*, 1994).

Potential function of the 50 kDa maize MAP

The 50 kDa maize MAP shares many properties with the P_{100} protein, such as the ability to co-assemble with endogenous microtubules (Figures 1 and 2), the ability to bind to neurotubules (Figure 3), and the presence of epitopes recognized by the anti-P_{100} and the anti-τ antibodies (Figure 6). However, the differences in cellular localization (Figure 8) point towards differential roles for the two proteins. Although the P_{100} protein did not co-localize with cortical microtubules, the co-assembly with endogenous tubulin (Figures 1 and 2), and the binding to neurotubules (Figure 3) suggest that the P_{100} protein has a MAP-function. The localization of the P_{100} protein to the nucleus could be related to the microtubule-nucleating activity of this organelle (Stoppin *et al.*, 1994). It is unlikely that the link between the P_{100} protein and tubulin is caused by unspecific co-precipitation, since both the binding to neurotubules as well as the co-assembly with endogenous tubulin withstood 0.15 M KCl, and high concentrations of salt were needed to release the P_{100} protein from the microtubule pellet again. Moreover, the transition from the soluble to the sedimented fractions and vice versa was nearly quantitative (Figures 1–4).

In contrast to the P_{100} protein, the 50 kDa maize MAP seems to be rather related to the state of cortical microtubules. The observation that the cortical microtubules in the elongating tissue appear to be arranged in bundles (Figure 8c–e) that are easily stained compared with the fine network, which is difficult to visualize (Figure 8a), observed in etiolated cells, makes it interesting to ask, whether the function *in vivo* of the 50 kDa maize MAP is to bundle microtubules, such as has been found for the neural MAP τ (Kanai *et al.*, 1989) or homologues of the elongation factor EF1-α (Cyr and Palevitz, 1989; Hasezawa and Nagata, 1993). However, despite the similar molecular weights (50 kDa), the preliminary sequence data do not show any relation of the 50 kDa MAP to EF1-α. Alternatively, it might stabilize the transverse orientation of cortical microtubules which seems to be essential for repartitioning growth from transverse expansion towards elongation (Giddings and Staehelin, 1991; Green, 1980). A third possibility might be a function of the 50 kDa maize MAP in the microtubule-organizing centres that seem to be important for the control of cell shape (Falconer *et al.*, 1988; Hasezawa and Nagata, 1993; Marc and Palevitz, 1990). Future work will be directed at examining those possibilities.

Experimental procedures

Plant material

Caryopses of maize (*Zea mays* L. cv. BRIO42.HT, Asgrow, Bruchsal, Germany) were soaked in de-ionized water for 2 h and then sown, embryo up, for 3 days either in complete darkness (cell elongation inhibited) or under continuous far-red light (1 μmol m^{-2} sec^{-1}) inducing phytochrome-dependent cell elongation (Briggs, 1963a). To induce the phytochrome system, a schedule with continuous far-red light was used instead of red-light pulses, because by such a treatment maximal photomorphogenetic activity of the phytochrome system could be achieved without induction of greening and photosynthesis (Mohr, 1972). Far-red light was isolated as described by Schäfer (1977). If not stated otherwise, coleoptiles, mesocotyls, and roots were harvested into liquid nitrogen under safe light (Schäfer, 1977).

Assay for neurotubule binding, co-assembly with endogenous tubulin, and heat stability

The tissue was ground in liquid nitrogen, until a fine powder was obtained. The frozen powder was then mixed with an equal volume of extraction buffer (25 mM Mes, 5 mM EGTA, 5 mM $MgCl_2$, 1 M glycerol, 1 mM GTP, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride, 1 mM aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin, pH 6.9) and centrifuged, immediately after thawing, at 60 000 ***g*** for 20 min at 4°C. The pellet was discarded and the supernatant further centrifuged at 100 000 ***g*** for 30 min at 4°C. The resulting supernatant was defined as cytosolic extract and either analysed directly or used for the various assays.

For the assay for co-assembly with endogenous tubulin (Vantard *et al.*, 1991), the cytosolic extract was incubated with 20 μM taxol in the presence of 1 mM GTP and 0.15 M KCl for 1 h at 27°C and then centrifuged at 60 000 ***g*** for 1 h at 27°C through a 20% sucrose cushion in extraction buffer. The pellet consisted of microtubules (Figure 1c). Thus, microtubule-associated proteins should remain with the pellet (fraction P_2), leaving unbound protein in the supernatant (fraction S_2). The microtubule pellet was resuspended in a small volume of extraction buffer containing 0.7 M KCl and again centrifuged through a sucrose cushion as described above (fractions S_3, defined as a MAP-fraction and P_3, the microtubule pellet consisting of tubulin). The electron microscopical images were obtained as described in Vantard *et al.* (1991).

For the neurotubule-binding assay, MAP-free neurotubulin was prepared as described by Vantard *et al.* (1994). Neurotubules were preformed and stabilized by taxol: for each millilitre of cytosolic extract, 0.5 mg MAP-free neurotubulin were incubated with 1 mM GTP and 20 μM taxol (Fluka, Neu-Ulm, Germany) in extraction buffer for 30 min at 30°C and carefully mixed with the cytosolic extract. The mixture was kept 15 min on ice in the presence of 20 μM taxol, 1 mM GTP, and 0.15 M KCl, and then treated as

described above for the co-assembly assay, with the difference that the temperature was kept at 4°C.

For the heat-stability assay thermostable fractions were prepared from the cytosolic extract as described in Vantard *et al.* (1994). For partial purification, the thermostable fractions from etiolated and from light-treated mesocotyls were subjected to anion-exchange chromatography on Quick Flow Sepharose Q (Pharmacia, Freiburg; Germany). The fractions were mixed with the equilibrated sepharose at a ratio of 1 volume fraction to 3 volumes sepharose and incubated for 15 min at 4°C. Then the column (15 mm diameter, 45 mm height) was poured and eluted, under continuous monitoring of the elution profile, at a flow rate of 2 ml min^{-1} with extraction buffer without protein inhibitors at pH 6.9. The concentration of the eluent (KCl) was increased in steps of 0.05 M. For each step, the column was eluted, until no further protein came off the column, before the concentration of KCl was raised further. P_{100} eluted between 0.35 and 0.4 M KCl, whereas the 50 kDa protein eluted between 0.25 and 0.3 M KCl.

Proteins were analysed on conventional SDS–polyacrylamide slab gels (7.5% w/v acrylamide and 0.2% w/v bisacrylamide) and subjected to immunoblotting as described in Vantard *et al.* (1994). Protein gels were stained with silver according to Ansorge (1982).

Immunofluorescence

Immunofluorescence labelling of cytoskeletal proteins was performed as described in detail in Toyomasu *et al.* (1994), using a mouse anti-β-tubulin antibody (Amersham, Little Chalfont, UK) and simultaneously a rabbit antibody raised against the 100 kDa heat-stable protein from maize cell cultures (Vantard *et al.*, 1994) as primary antibodies. Double fluorescence-labelling was achieved by a fluorescein-isothiocyanate-labelled anti-mouse-IgG antibody and a rhodamin-labelled anti-rabbit-IgG antibody. As controls, the antibody directed against the 100 kDa heat-stable protein was replaced by the respective pre-immune serum. To check the specificity of the nuclear label by this antibody (Figure 7), a control was included, where only this antibody was omitted. This type of control was always completely negative (data not shown). It should be mentioned, however, that unspecific staining of starch grains was observed in light-grown tissue (Figure 8c), even with secondary antibody alone. They could be distinguished from nuclei by shape, size and number. Moreover, these starch grains did not stain with DAPI.

Subcellular fractionation

For the isolation of nuclear extracts, the tissue was ground with liquid nitrogen and then incubated for 1 h at 25°C with 5 volumes of digestion buffer (0.7 M mannitol, 10 mM Mes, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.1 w/v bovine serum albumin, pH 5.8) containing 3% cellulase and 0.6% macerozyme. After centrifugation (3000 ***g***, 5 min, 4°C), the pellet was resuspended in washing buffer (0.25 M sucrose, 50 mM NaCl, 50 mM Mes, 25 mM EDTA, 0.1 % Triton X100, pH 6) and recentrifuged (3000 ***g***, 5 min, 4°C). This washing step was repeated twice. The resuspended pellet was then homogenized by shearing force (Ultra-Turrax) on ice and filtered through a 10 μm mesh. Nuclei were collected at 5000 ***g*** for 5 min at 4°C, and their purity and their integrity controlled by epifluorescence microscopy after staining with DAPI. Most of the nuclei (about 90%) appeared to be intact and only a few were disrupted by the extraction method used. The major contaminants in the preparation were amyloplast fragments. Membranous fractions enriched for plasma membranes were obtained using the aequous two-phase partitioning method described in detail in Widell and Larsson (1981). The activity of cytochrome C-oxidase was assayed as a marker for mitochondrial membranes, and the activity of cytochrome C-reductase as a marker for the endoplasmatic reticulum. Both activities were measured in a spectroscopical test (Lützelschwab *et al.*, 1988). Specific binding of tritium-labelled 1-*N*-naphthylphthalic acid amide served as a marker for the plasma membranes (Jacobs and Hertel, 1978). Purified fractions consisted of more than 90% of plasma membrane as deduced from the strong decline in the activity of cytochrome C-oxidase and cytochrome C-reductase in relation to the sharp increase of specific binding of 1-*N*-naphthylphtalic acid amide in the course of the purification protocol (data not shown).

Antibodies

Mouse anti-β-tubulin and anti-α-tubulin (Amersham, Little Chalfont, UK) was used at a dilution of 1:1000 for immunofluorescence staining and at 1:5000 for immunoblotting. The rabbit anti-P_{100} IgG (Vantard *et al.*, 1994) was used at 1:1000 for immunofluorescence staining, and at 1:5000 to 1:20 000 for immunoblotting. Peroxidase-conjugated anti-rabbit-IgG (Sigma, Neu-Ulm, Germany) was used as secondary antibodies for immunoblotting at 1:2500. Rhodamine-labelled anti-rabbit-IgG, and fluorescein-isothiocyanate labelled anti-mouse-IgG (both purchased from Amersham, Little Chalfont, UK) were applied at 1:50 for immunofluorescence staining. The affinity-purified rabbit anti-τ antibodies were kindly provided by A. Fellous (Vantard *et al.*, 1991).

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References

Ansorge, W. (1982) Fast visualization of protein bands by impregnation in potassium permanganate and silver nitrate. *Electrophoresis,* **82**, 235–242.

Blancaflor, E.B. and Hasenstein, K.H. (1993) Organization of cortical microtubules in graviresponding maize roots. *Planta,* **191**, 231–237.

Briggs, W.R. (1963a) Mediation of phototropic response of corn coleoptiles by lateral transport of auxin. *Plant Physiol.* **38**, 237–247.

Briggs, W.R. (1963b) Red light, auxin relationships, and the phototropic responses of corn and oat coleoptiles. *Am. J. Bot.* **50**, 196–207.

Cande, W.J. (1990) Centrosomes: composition and reproduction. *Curr. Opinions Cell Biol.* **2**, 301–305.

Cyr, R.J. (1994) Microtubules in plant morphogenesis: role of the cortical arrays. *Ann. Rev. Cell Biol.* **10**, 153–180.

Cyr, R.J. and Palevitz, B.A. (1989) Microtubule-binding proteins from carrot. I. Initial characterization and microtubule bundling. *Planta,* **177**, 254–260.

Durso, N.A. and Cyr, R.J. (1994) A MAP by any other name would still bind to microtubules. *Plant Cell,* **6**, 1699–1702.

Falconer, M.M., Donaldson, G. and Seagull, R.W. (1988) MTOCs in higher plant cells: an immunofluorescent study of microtubule

assembly sites following depolymerization by APM. *Protoplasma,* **144**, 46–55.

Furuya, M. (1993) Phytochromes: their molecular species, gene families, and functions. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 617–645.

Giddings, T.H. and Staehelin, A. (1991) Microtubule-mediated control of microfibril deposition: a re-examination of the hypothesis. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed.). London: Academic Press, pp. 85–99.

Green, P.B. (1980) Organogenesis–a biophysical view. *Ann. Rev. Plant Physiol.* **31**, 51–82.

Hasezawa, S. and Nagata, T. (1993) Microtubule organizing centers in plant cells: Localization of a 49 kDa protein that is immunologically cross-reactive to a 51 kDa protein from sea urchin centrosomes in synchronized tobacco BY-2 cells. *Protoplasma,* **176**, 64–74.

Jacobs, M. and Hertel, R. (1978) Auxin binding to subcellular fractions from *Cucurbita* hypocotyls: in vitro evidence for an auxin transport carrier. *Planta,* **142**, 1–10.

Kreis, T. and Vale, R. (1993) *Guidebook to the Cytoskeletal and Motor Proteins.* Oxford: Oxford University Press, pp. 101–105.

Kutschera, U. (1991) Regulation of cell expansion. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed.). London: Academic Press, pp. 149–158.

Lambert, A.-M. (1993) Microtubule-organizing centers in higher plants. *Curr. Opin. Cell Biol.* **15**, 116–122.

Lee, G. (1993) Non-motor microtubule associated proteins. *Curr. Opinions Cell Biol.* **5**, 88–94.

Lützelschwab, M., Asard, H. Ingold, U. and Hertel, R. (1988) Heterogeneity of auxin-accumulating membrane vesicles from *Cucurbita* and *Zea*: a possible reflection of cell polarity. *Planta,* **177**, 304–311.

Marc, J. and Palevitz, B.A. (1990) Regulation of the spatial order of cortical microtubules in developing guard cells in *Allium. Planta,* **182**, 626–634.

Matus, A. (1990) Microtubule-associated proteins. *Curr. Opinions Cell Biol.* **2**, 10–14.

Mohr, H. (1972) *Lectures on Photomorphogenesis.* Berlin: Springer Verlag.

Morejohn, L.C. (1994) Microtubule binding proteins are not necessarily microtubule-associated proteins. *Plant Cell,* **6**, 1696–1699.

Nick, P. and Furuya, M. (1992) Induction and fixation of polarity–Early steps in plant morphogenesis. *Dev. Growth Differ.* **34**, 115–125.

Nick, P., Bergfeld, R., Schäfer, E. and Schopfer, P. (1990) Unilateral reorientation of microtubules at the outer epidermal wall during photo- and gravitropic curvature of maize coleoptiles and sunflower hypocotyls. *Planta,* **181**, 162–168.

Nick, P., Yatou, O., Furuya, M. and Lambert, A.-M. (1994) Auxin-dependent microtubule responses and seedling development are affected in a rice mutant resistant to EPC. *Plant J.* **6**, 651–663.

Pickard, B.G. (1985) Early events in geotropism of seedling shoots. *Ann. Rev. Plant Physiol.* **36**, 55–75.

Pjon, Ch.-J. and Furuya, M. (1967) Phytochrome action in *Oryza sativa* L. I. Growth responses of etiolated coleoptiles to red, far-red and blue light. *Plant Cell Physiol.* **8**, 709–718.

Rothert, K. (1894) Über heliotropismus. Breslau: Kern Verlag, pp. 25–27, 43–47.

Schäfer, E. (1977) Kunstlicht und Pflanzenzucht. In *Optische Strahlungsquellen* (Albrecht, H., ed.). Grafenau, Germany: Lexika Verlag, pp. 249–266.

Shibaoka, H. (1991) Microtubules and the regulation of cell morphogenesis by plant hormones. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W. ed.). London: Academic Press, pp.159–168.

Solomon, F., Magendantz, M. and Salzman, A. (1979) Identification with cellular microtubules of one of the co-assembling microtubule-associated proteins. *Cell,* **18**, 431–438.

Stoppin, V., Vantard, M., Schmit, A.-C. and Lambert, A.-M. (1994) Isolated plant nuclei nucleate microtubule assembly: the nuclear surface in higher plants has centrosome-like activity. *Plant Cell,* **6**, 1099–1106.

Toyomasu, T., Yamane, H., Murofushi, N. and Nick, P. (1994) Phytochrome inhibits the effectiveness of gibberellins to induce cell elongation in rice. *Planta,* **194**, 256–263.

Vantard, M., Levilliers, N., Hill, A.M., Adoutte, A. and Lambert, A.-M. (1990) Incorporation of *Paramaecium* axonemal tubulin into higher plant cells reveals functional sites of microtubular assembly. *Proc. Natl Acad. Sci. USA,* **87**, 8825–8829.

Vantard, M., Schellenbaum, P., Fellous, A. and Lambert, A.-M. (1991) Characterization of maize microtubule-associated proteins, one of which is immunologically related to tau. *Biochemistry,* **30**, 9334–9340.

Vantard, M., Peter, C., Fellous, A., Schellenbaum, P. and Lambert, A.-M. (1994) Characterization of a 100 kDa heat-stable microtubule-associated protein from higher plants. *Eur. J. Biochem.* **220**, 847–853.

Wiche, G., Oberkanins, C., and Himmler, A. (1991) Molecular structure and function of microtubule-associated proteins. *Int. Rev. Cytol.* **124**, 217–273.

Widell, S. and Larsson, C. (1981) Separation of presumptive plasma membranes from mitochondria by partition in an aequous two-phase system. *Physiol. Plant.* **51**, 368–374.

Zandomeni, K. and Schopfer, P. (1993) Reorientation of microtubules at the outer epidermal wall of maize coleoptiles by phytochrome, blue-light photoreceptor and auxin. *Protoplasma,* **173**, 103–112

Zhang, D., Waldsworth, P. and Hepler, P.K. (1990) Microtubule dynamics in living dividing plant cells: confocal imaging of microinjected fluorescent brain tubulin. *Proc. Natl Acad. Sci. USA,* **87**, 8820–8824.