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Developmental and light-dependent changes of the cytosolic chaperonin containing TCP-1 (CCT) subunits in maize seedlings, and the localization in coleoptiles

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Summary

The cytosolic chaperonin containing TCP-1 (CCT) is known to keep fold cytoskeletal proteins and is involved in the proper organization of the cytoskeleton. These studies are based on the assumption that growth responses linked to structural rearrangement of the plant cytoskeleton include the action of CCT and the need for newly synthesized tubulin. The presence of the α- and ε- subunits of CCT was investigated in soluble fractions of protein extracts from maize mesocotyls and coleoptiles at distinct growth stages. The CCT-subunits, tubulins and actin decreased in the coleoptile in response to far-red light. In addition, independent from light treatment, the amount of CCTε abundance declined with age in coleoptiles and mesocotyls between 2 and 4.5 days after sowing. In contrast to CCTε, no significant light regulation of CCTα was found in the mesocotyl. In two day old, light-grown rapidly elongating coleoptiles part of the CCTα subunit and the bulk of actin and tubulin was found shifted into fractions of high molecular weight complexes when compared to slowly elongating, dark grown coleoptiles. In 4.5 day old, etiolated and elongating coleoptiles, part of both CCT-subunits and cytoskeleton proteins were found in fractions of high molecular weight. A complete disappearance of these polypeptides was observed in old far-red irradiated growth-arrested coleoptiles. CCTε was found to be co-localized to microtubular structures and to the nucleus. We conclude from our data that abundance of CCT-subunits in soluble extracts is dependent on age and light treatment, but independent from the growth stage of mesocotyl and coleoptile.

Introduction

The chaperonin containing TCP-1 (CCT; Kubota *et al.*, 1994), also named TCP-1 Ring Complex (TRiC, Frydman *et al.*, 1992), occurs in the eukaryotic cytosol and has been recently characterized as the machinery responsible for folding of actin and tubulin *in vitro* and *in vivo* (Lewis *et al.*, 1992; Sternlicht *et al.*, 1993; Yaffe *et al.*, 1992). The *in vitro* folding studies demonstrated the interaction of CCT with components of the cytoskeleton such as β-actin, α-, β-, and γ-tubulin (Gao *et al.*, 1992; Gao *et al.*, 1993) and vertebrate actin-related protein (RPV; Melki *et al.*, 1993). Recent studies on the folding pathway of β-tubulin starting from denatured substrate bound to CCT shows a highly controlled sequence of interactions between folding intermediates produced by CCT and four cofactors (Tian *et al.*, 1996), which is distinct from the known interactions of the GroEL/GroES system with unfolded substrates (Hartl, 1996). *In vitro* translated actin interacts with hsp40/hsc70 shortly after emergence from the ribosome before transfer to CCT (Frydman and Hartl, 1996).

In yeast, a series of data indicates the importance of CCT for proper organization of microtubules. Different mutations in the CCTα gene led to cold- or thermosensitive yeast strains displaying cell-cycle arrest. (Miklos *et al.*, 1994; Ursic and Culbertson, 1991; Ursic *et al.*, 1994;). The yeast cultures contained high frequencies of binucleate or anucleate cells, a phenotype also found when the CCTβ-gene was mutagenized (Chen *et al.*, 1994; Miklos *et al.*, 1994). The results suggest a pertubation of nuclear division and migration with all of the mutations exhibiting partially disrupted structures of microtubules. Actin filaments were affected by mutations in the yeast genes of CCTα-, CCTβ-, CCTγ-, and CCTδ subunits (Chen *et al.*, 1994; Ursic *et al.*, 1994; Vinh and Drubin, 1994). These *in vivo* findings and recent data on the presence of CCT in centrosomes of mammalian cells and their role in microtubule nucleation (Brown *et al.*, 1996) indicate the participation of CCT in the organization of microtubular structures. This is of special interest in plants where cortical microtubules (cMTs) are one of their characteristic features. The orientation of cMTs is usually found to parallel newly deposited cellulose fibers and microtubules (MT) probably control the direction in which those microfibrils are placed in the cell wall (reviewed in Giddings and Williamson, 1991; Wymer and Lloyd, 1996). Because of their supposed templating activity, MTs are thought to play an important role in directional cell growth (Green, 1980; Shibaoka, 1991; Wymer and Lloyd, 1996).

Cortical MTs are found to reorientate rapidly in response to external stimuli such as light or gravity as well as to endogenous factors such as plant hormones. In epidermal

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cells of the maize coleoptiles, the rearrangement of cMTs can be triggered either by blue light, auxin, gravity (Nick *et al.*, 1990) or by the action of phytochrome (Zandomeni and Schopfer; 1993). If all newly synthesized actin and tubulin molecules are folded by CCT, this complex might play an important role in the reorganization of cortical microtubules in the plant cell. In addition, since CCT has at least eight subunits (Kubota *et al.*, 1995a; Rommelaere *et al.*, 1993), the question arises if CCT changes its stoichiometry of subunit composition during developmental processes to adapt to the physiological needs of growing tissues.

Our present study is based on the assumption that growth responses linked to structural rearrangement of the plant cytoskeleton include the need of newly formed tubulin and hence the action of CCT, which has been characterized as an important factor for proper organization of the yeast cytoskeleton. We consequently began to examine the presence of CCT-subunits in relation to their potential substrates during phytochrome-controlled growth responses of young maize seedlings. Our results show a strong decrease of the abundance of CCT-subunits, actin and tubulins in soluble extracts from irradiated coleoptiles. Furthermore, the association of CCT-subunits and cytoskeleton proteins to high molecular weight complexes is dependent from age and irradiation of the maize seedlings. We found no direct evidence for a link between the growth stage of coleoptiles and abundancy of CCT-subunits. The CCTε-subunit was found to be localized to the cell nucleus and to microtubular bundles of developing vessels.

Results

Specificity of antibody cross-reaction

The CCTε-subunit was detected in maize seedlings with an antibody raised against the homologous subunit from oat (Ehmann *et al.*, 1993). A comparison between the known plant CCTε subunits from oat as a monocotyledonous plant and the dicotyledon species cucumber (Ahnert *et al.*, 1996) exhibits 89% homology and 94.4% similarities at the level of the polypeptide sequence. We expect the relationship between oat and maize to be probably higher and the antibody used for CCTε detection very likely recognizes the correct subunit. In some of the Western blots shown (Figures 1b, 3–5) the CCTε antibody detects two bands of approximately 60 and 66 kDa apparent molecular weight. The signal at 60 kDa was not always found nor could it be correlated to certain physiological stages during seedling development. We assume the 60 kDa band is a partial degradation product of CCTε which appears during native protein extraction. The specificity of the CCTε-antibody was further confirmed by lack of cross-reaction with bacterially

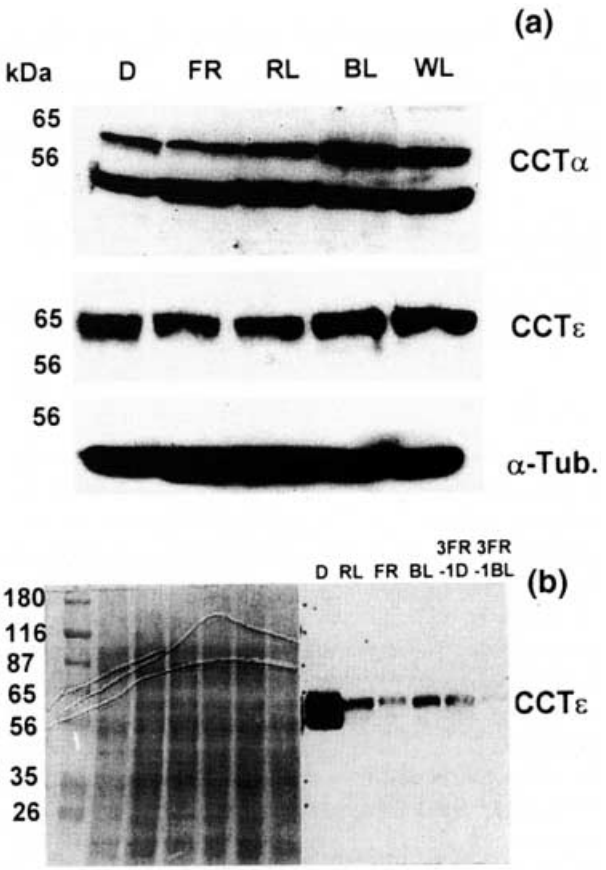

Figure 1. Western blot analysis using either total (a) or soluble extracts (b) from maize coleoptiles harvested at four days after sowing.
(a) Seedlings were either kept in dark (D) or grown in continuous far-red (FR), red (RL), blue (BL) or UV-containing white light (WL) prior to harvest. Extracts were separated on SDS–PAGE and analysed by Western blot performed using primary antibodies raised against CCTα, CCTε, and α-tubulin.
(b) The left-hand panel shows the gel stained with Coomassie, on the right-hand panel the corresponding Western blot using the CCTε-antibody. Note the one day dark period or one day BL treatment after three days far-red irradiation (3 far-red-1D and 3dfar-red-1bL respectively). Size markers are positioned on the left side of the panels.

overexpressed CCTα, CCTβ, CCTδ and CCTζ subunits from yeast. Antisera raised against these yeast subunits did not cross-react with bacterially overexpressed full-size oat CCTε (data not shown).

In our study we used the CCTα-antiserum raised against the yeast subunit to check for changes in the abundance ratio of different subunits during seedling development. This antiserum detects a polypeptide of approximatly 63 kDa apparent molecular weight which coincides with the size of bacterially overexpressed yeast CCTα protein (data not shown). Yeast CCTα has a calculated molecular mass of 60.480 kDa, the CCTα subunit from *Arabidopsis* a calculated molecular mass of 59.229 kDa (Mori *et al.*, 1992). The yeast CCTα-antiserum also cross-reacts strongly with two polypeptides in the range of 50 kDa (Figures 1a, 2a and b). These proteins can be separated from the 60 kDa

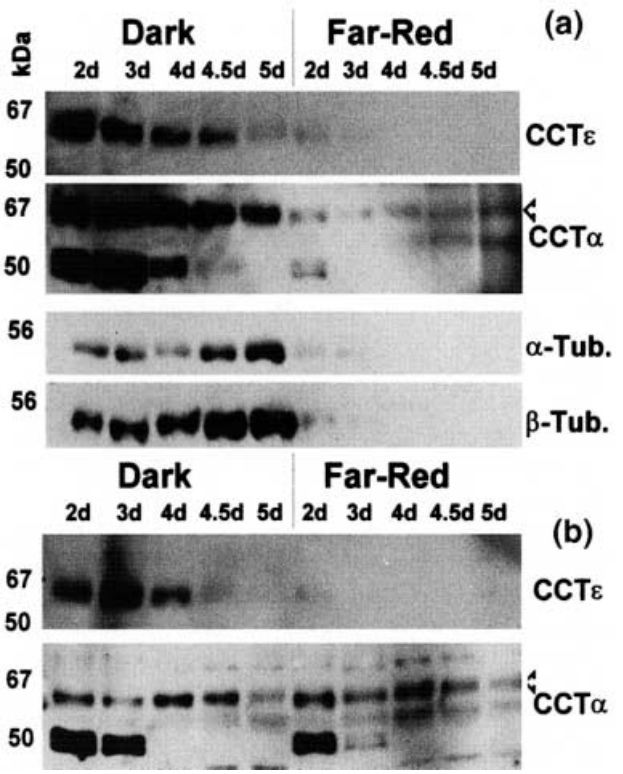

Figure 2. Time course of CCTα, CCTε and tubulin abundance between two and five days after sowing from either coleoptiles (a) or mesocotyl (b) grown in darkness or under continuous far-red light.
For Western blot analysis, 12 μg soluble protein per lane were loaded on the SDS–PAGE. Arrowheads indicate position of CCTα.

polypeptide by ion exchange chromatography (Figure 3b) or sucrose gradient fractionation (Figure 4b–d) suggesting that they are not components of the CCT complex.

Light-dependence of protein abundance of CCTα, CCTε and α-tubulin in total and soluble extracts from the maize coleoptile

Any light treatment stimulates elongation of coleoptiles at early stages of maize development and simultaneously inhibits growth of the mesocotyl. In the following experiments, wave-length dependency of protein abundance was examined possibly linked to the growth stage of the tissues. Western analysis of total extracts taken from coleoptiles grown for four days either in the dark (D) or different light qualities revealed no significant light regulation of CCTα, CCTε and α-tubulin abundance (Figure 1a). In contrast, a drastic decrease of CCTε abundance occurs in the soluble part of the protein extracts in response to any light treatment (Figure 1b). We therefore focused on the dynamics of CCT-subunits and cytoskeletal components in soluble extracts of maize seeedlings.

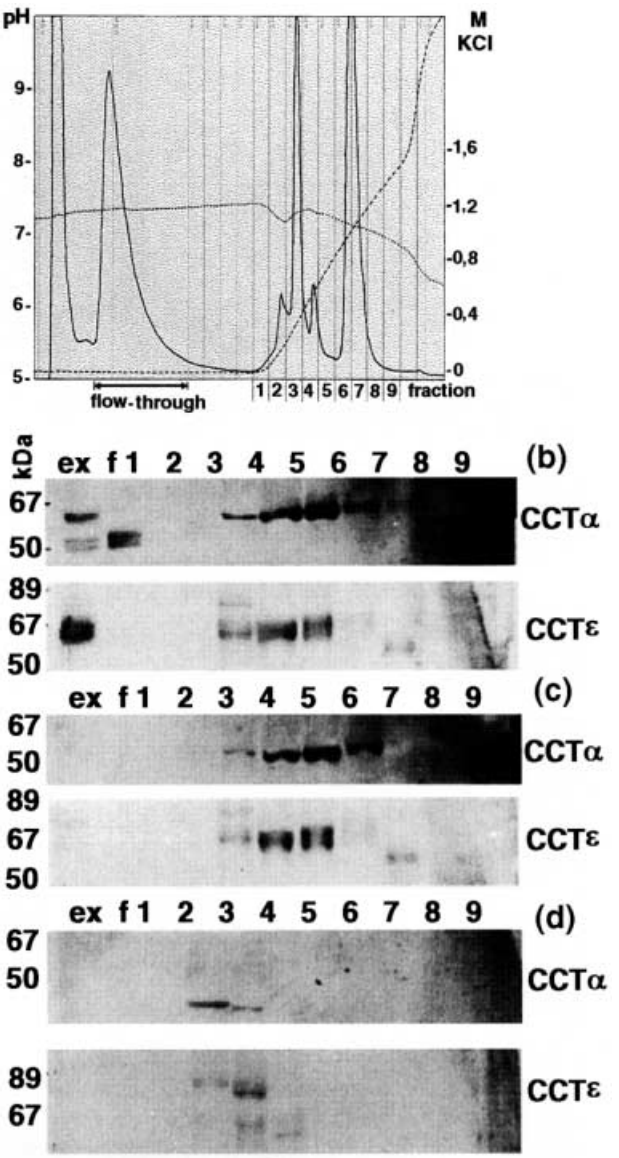

Figure 3. Anion exchange chromatographie of soluble extracts from coleoptiles that were grown for two days in darkness (b), two days under far-red light (c), or 4.5 days under far red-light (d).
Gels were loaded with soluble extract (ex), flow-through (f) and nine fractions (1–9) obtained over a 0–1.5 M KCl gradient run on a HQ20 column (see Experimental procedures). (a) Typical elution profile of fractions obtained with an extract from two day old, dark-grown coleoptiles.

Development- and light-dependent abundance of CCTα, CCTε and tubulin in soluble extracts from maize seedlings

Since growth responses in plants are usually accompanied by a rearrangement of microtubular structures, we examined if a correlation exists between elongation of the mesocotyl and coleoptile and the presence of CCT-subunits and tubulins in soluble extracts. Both organs respond to light with opposite growth responses as mentioned in the previous section. In the coleoptile, the growth response is

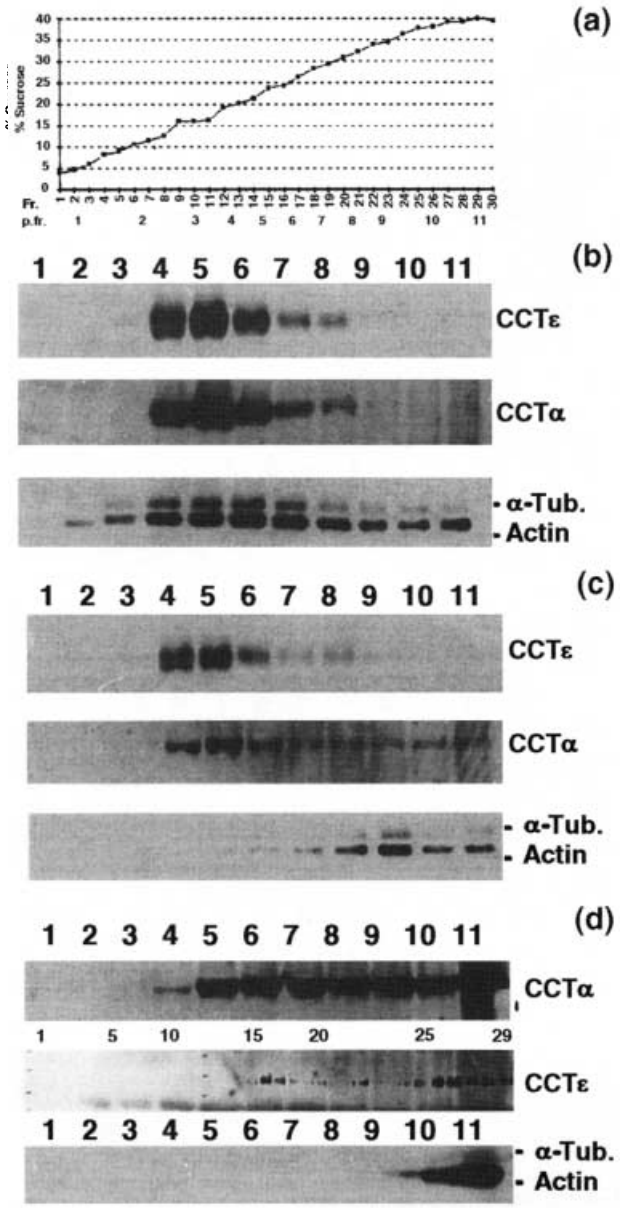

Figure 4. Fractionation by sucrose gradient centrifugation of soluble extracts from coleoptiles of either etiolated, two day old (b), etiolated 4.5 day old (d) or two day old far-red-grown (c) maize seedlings.
(a) Sucrose gradient determined for extracts from two day old etiolated coleoptiles. The 30 fractions (Fr) collected are indicated along the X-axis with the pattern of pooled fractions 1–11 (p. Fr) shown below. The fractions were subjected to SDS–PAGE and Western blot analysis. The middle panel in (d) shows the detection of CCTε with unpooled fractions from the sucrose gradient.

based exclusively on changes in cell elongation, whereas changes in cell number occur in the mesocotyl (F. Waller and P. Nick, unpublished observations).

Soluble extracts were prepared from maize coleoptiles and mesocotyls grown either in continuous dark or under continuous far-red light for 2–5 days after sowing and analysed by SDS–PAGE and Western blotting. The abundance of CCTε decreased with increasing age of both, dark-grown coleoptiles (Figure 2a) and mesocotyls (Figure 2b). In far-red light a drastic decrease of CCTε in both organs was observed as early as 2 days after sowing, becoming more pronounced throughout the whole time course (coleoptile: Figure 2a; mesocotyl: Figure 2b) consistent with the results shown in Figure 1(b).

The amount of CCTα decreased slightly with age in dark-grown coleoptiles (Figure 2a, Dark), but remained more or less constant in mesocotyls during the time course taken (Figure 2b, Dark). Similar to CCTε, CCTα disappears in soluble extracts from light-treated coleoptiles (Figure 2a far-red), but not in the mesocotyl (Figure 2b, far-red). The putative *in vivo* substrates of CCT, α- and β-tubulin, became more abundant with time in soluble extracts of etiolated coleoptiles (Figure 2a), but were found to be strongly depleted in far-red light-grown coleoptiles, a finding also observed for actin (data not shown). Thus, CCT-subunits and tubulins strongly decrease with parallel time courses in the far-red grown coleoptile. This light regulation is overlaid by a developmental control of CCTε abundance in soluble extracts from both organs. The data suggest that CCT is a dynamic complex in soluble extracts with a possibly varying composition of subunit types. Interestingly, the 50 kDa proteins recognized by the CCTα-antisera (Figures 1 and 2) are abundant at early stages only and decrease with time in etiolated coleoptiles and mesocotyls, with far-red-light inducing a further reduction (Figure 2a). These proteins were also strongly recognized by antisera raised against the yeast CCTδ-subunit and, very weakly, by antisera against CCTβ (data not shown).

Fractionation of soluble protein extracts by perfusion anion-exchange chromatography

The results from the previous experiments showed distinct time points when the ratio of CCTα and CCTε content was clearly altered and the coleoptiles passed through distinct growth stages (see below). In order to characterize the properties of CCTα- and CCTε-containing complexes, soluble extracts were prepared from coleoptiles either grown in the dark or far-red light for 2 or 4.5 days and fractionated by anion exchange chromatography. The elution profiles of proteins (Figure 3a) was found to be identical for all four extracts and nine fractions each were sampled covering the whole gradient from 0 to 1.5 M KCl. For Western blot analysis, the total soluble extract, the flow-through and the nine fractions were subjected to separation on SDS–PAGE (Figures 3b–d). CCTε and CCTα were found in Fractions 3 to 5 in soluble extracts from two day old coleoptiles grown in the dark (Figure 3b) or in far-red light (Figure 3c). In contrast to CCTε, CCTα was clearly detectable in Fraction 6. Hence, despite the light-dependent drastic decrease of

both subunits in the total soluble extract (Figures 1b, 2a and 3b, c, 'ex'), this reduction could not be observed in the fractions from the anion exchange column (compare Figure 3b and c). The 50 kDa proteins recognized by the CCTα-antisera remained in the unbound fraction (Figure 3b, 'f') and were not detected in extracts from far-red-treated tissues (Figure 3c, 'f'), as shown in Figure 2. In extracts from etiolated 4.5 day old coleoptiles, the detection pattern of CCT-subunits was similar to that for younger tissues (data not shown, Figure 3b). None of the CCT-subunits could be detected in fractions taken from soluble extracts of 4.5 day old, irradiated coleoptiles (Figure 3d). Solely unspecific signals with bands of higher (CCTε) or lower apparent molecular weight (CCTα) became visible after prolonged exposition of the membranes (Figure 3d).

Fractionation of soluble protein extracts by sucrose gradient centrifugation

Current purification protocols of CCT complex include the separation of extracts on a sucrose gradient (Frydman *et al.*, 1992; Lewis *et al.*, 1992; Yaffe *et al.*, 1992) which allow the separation and detection of high molecular weight complexes. CCT sediments as a particle of 20S size and is found in fractions ranging from 19 to 21% sucrose density. Soluble extracts were prepared from coleoptiles grown in either the dark or under far-red light for two or 4.5 days, then fractionated by centrifugation on a linear sucrose gradient. Thirty fractions were collected from each of the four gradients (Figure 4a). The fractions were pooled as indicated in the gradient graph (Figure 4a) and subjected to Western blot analysis (Figure 4b–d). Taking extracts from two day old etiolated coleoptiles, the bulk of CCTε and CCTα was detected in fractions 4 to 6 corresponding to 18–23% sucrose density (Figure 4b). Samples from two day old, far-red treated coleoptiles exhibit a similar distribution for CCTε, but CCTα was found now in fractions of higher sucrose densities as well (Figure 4c). In etiolated tissues of two day old coleoptiles, most α-tubulin and actin were detected in the same fractions as CCTε and CCTα (Figure 4b). In two day old irradiated coleoptiles, these polypeptides were found in fractions of higher sucrose density as some parts of CCTα. (Figure 4c). Thus, despite the strong far-red-light dependent decrease of tubulin and actin in soluble extracts (Figures 1 and 2), these proteins were enriched and could be clearly detected. Taking extracts from 4.5 day old etiolated coleoptiles, CCTα was present also in fractions of up to 40% sucrose (Figure 4d), in contrast to two day old coleoptiles (Figure 4b). As was the case for CCTα, CCTε is shifted towards high sucrose densities (Figure 4d) when compared to the situation in young coleoptiles (Figure 4b). A very pronounced change occurred for the distribution of actin and tubulin which were exclusively found at highest sucrose densities (Figure 4d). In particular, α-tubulin seemed to be present in these fractions in minor amounts. In extracts of 4.5 day far-red grown coleoptiles, Western blots showed no signals with any of the polypeptides tested (data not shown) confirming the results shown in Figure 2.

Taken together, the results demonstrate a cofractionation for CCT-subunits and most part of the putative folding substrates in young (two day old) etiolated coleoptiles. Upon irradiation of coleoptiles with far-red-light, α-tubulin, actin and part of CCTα, but not CCTε, were found to sediment into fractions of high sucrose densities. In older (4.5 day old) etiolated coleoptiles, part of both CCT-subunits became sedimentable into high sucrose densities with an extreme shift in case of tubulin and actin. None of the polypeptides were found in case of older, irradiated tissues.

Co-immunoprecipitation of CCTε subunits using a monoclonal antibody directed against β-tubulin

The co-sedimentation of actin and tubulin with CCT-subunits in sucrose gradient fractions does not necessarily prove a true interaction between the cytoskeletal components and CCT. Hence, a co-immunoprecipitation was performed to obtain further evidence for direct interaction of CCT and one of its substrates. The soluble extracts from etiolated or far-red treated, four day old coleoptiles were assayed using a monoclonal antibody raised against β-tubulin. The precipitates were separated by SDS–PAGE and CCTε was detected by Western blot analysis (Figure 5). Similar to the experiment shown before (e.g. Figure 1b), a drastic light-dependent decrease of CCTε is seen in soluble extracts (Figure 5a, lane 1 (dark) versus lane 2 (far-red)). CCTε is enriched in the pellet after immunoprecipitation in soluble extracts from etiolated coleoptiles (Figure 5a, lane 3) with no residual subunit detected in the supernatant (Figure 5a, lane 4). In contrast, no CCTε is found in the pellet of extracts from irradiated tissues, (Figure 5a, lane 5), but residual amounts of CCTε were found in the supernatant (Figure 5a, lane 6). The CCTε-antibody does not show any cross-reactions with the monoclonal antibody used for immunoprecipitation (Figure 5a, lane 7). As a further control, when normal serum from mouse was used for immunoprecipitation instead of the monoclonal β-tubulin antibody, CCTε remained in the supernatant (Figure 5b).

These data confirm the previous findings demonstrating the decrease of CCTε and β-tubulins from soluble extracts (see Figure 1), indicating that CCTε is likely to be a part of the CCT complex bound to β-tubulin. Precipitation of CCTε had obviously been complete when extracts from etiolated coleoptiles were used since no signal was found in the supernatant. In contrast, no CCTε was found in the pellet using samples from far-red-irradiated tissues. A weak signal detected in the supernatant fraction (Figure 5a, lane 6) suggests either the complete disappearance of β-tubulin

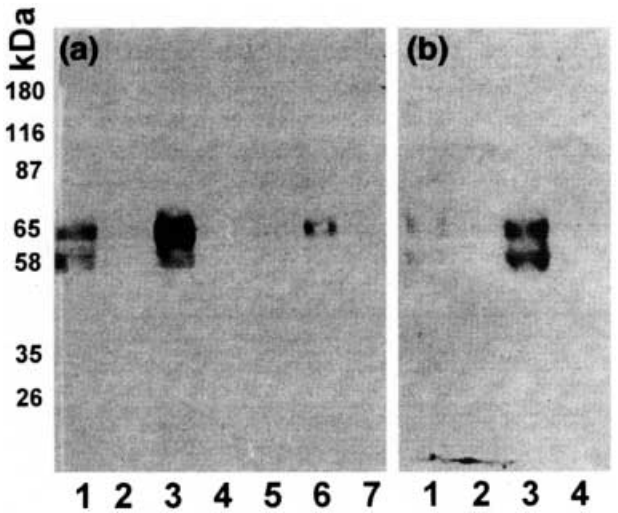

Figure 5. Co-immunoprecipitation of CCTε by a monoclonal antibody against β-tubulin.
Blots shown in (a) and (b) were probed with the CCTε antibody. (a) Lanes 1 and 2: soluble extracts prepared from either dark-grown or far-red-irradiated coleoptiles respectively harvested at four days after sowing. Lanes 3 and 5: immunoprecipitates of extracts from etiolated or irradiated coleoptiles. Lanes 4 and 6: supernatant of the same extracts after immunoprecipitation. Lane 7: β-Tubulin antibody used for precipitation. (b) Control experiment using mouse normal sera instead of β-tubulin antibody for precipitation. Lane 1 and lane 2: immunoprecipitates precipitates obtained from samples of either etiolated or far-red irradiated tissues respectively. Lanes 3 and 4: supernatant of the samples as mentioned before. CCTε remains in the supernatant of dark-extract (Lane 3).

from soluble extracts of irradiated tissues or an altered mode of interaction between CCTε and β-tubulin, since most of the subunit remained in the supernatant.

Localization of CCTε and tubulin in coleoptile tissues

The distribution of CCTε was examined by indirect double-stain immunfluorescence of microtubules and CCTε using confocal laser scanning microscopy. Figures 6a and b show the same cells immunedecorated with antibodies against α- and β-tubulin (Figure 6a) and the CCTε-antibody (Figure 6b). Figure 6(a) shows the typical pattern of cortical microtubules within subepidermal cells of four day old, etiolated coleoptiles. Using the CCTε antibody, a patchy staining pattern was obtained which could be aligned to cMTs in certain cases (Figure 6a, b, arrows), but was very often found not to co-localize with a cortical microtubule (arrowheads). In developing vessels, CCTε was found, again in a patchy pattern (Figure 6d), associated with bundled cMTs that are found at sites of developing wall thickenings in cells of the protoxylem (Figure 6c). This co-localization is increasingly impaired in further developing vessels due to progressive lignification of the cell reinforcement structures that finally cause strong autofluorescence (data not shown). CCTε was also found to decorate the nuclear envelope as shown in Figure 6(e), (f) and (g). As a control, a nucleus staining dye was used in parallel (Figure 6h). The specificity of the staining pattern was confirmed by negative controls using appropriate normal sera instead of primary antibodies.

Discussion

Physiology of growth responses during development of the maize seedling

In the maize coleoptile, cell elongation is stimulated by light via the photoreversible phytochrome system and is accompanied by a reorientation of cMTs in epidermal cells into transverse arrays (Zandomeni and Schopfer, 1993). In contrast, the growth of the mesocotyl is inhibited by light with the response also being phytochrome-dependent. The cMTs of cells in maize mesocotyl display a reorientation into longitudinal arrays (P. Nick, unpublished results). During our analysis, we focused on two time points: at two days after sowing, dark-grown coleoptiles have not entered into elongation phase, whereas far-red-irradiated coleoptiles exhibit rapid elongation. At 4.5 days after sowing, elongation is gradually developing in dark-grown coleoptiles, whereas in far-red grown seedlings the primary leaves already pierce the fully elongated coleoptiles. We assessed the possible correlation between these growth responses and the presence of CCT-subunits and cytoskeleton components that are putative *in vivo* folding substrates of CCT. Our studies are based on the assumption that growth responses which are linked to structural rearrangement of the plant cytoskeleton involve the action of CCT and the need for newly formed tubulin.

Light-induced sedimentability of CCT-subunits and cytoskeletal components

The analysis of total crude extracts from coleoptiles grown for four days gave no indication of light control of the proteins examined (Figure 1a). Rather, an age-dependent decrease of CCTα and CCTε was found when total extracts from two day and four day old coleoptiles were compared (data not shown). In contrast, in soluble extracts of coleoptiles, the presence of both subunits strongly decreases after far-red-light treatment and a clear decrease of CCTε with time (Figure 2a) was detected in addition. Such a temporal pattern of abundance is barely found for CCTα in coleoptiles (Figure 2a) and, furthermore, CCTα is not controlled by light in the mesocotyl. In contrast, CCTε shows a light- and age-dependent decrease in the mesocotyl (Figure 2b). These data suggest age- and light-dependent changes in the sedimentability of CCT-subunits and also in the amount of different CCT-subunits. This might indicate a regulation of the stoichiometry of subunit composition within the whole chaperonin population in soluble extracts (see

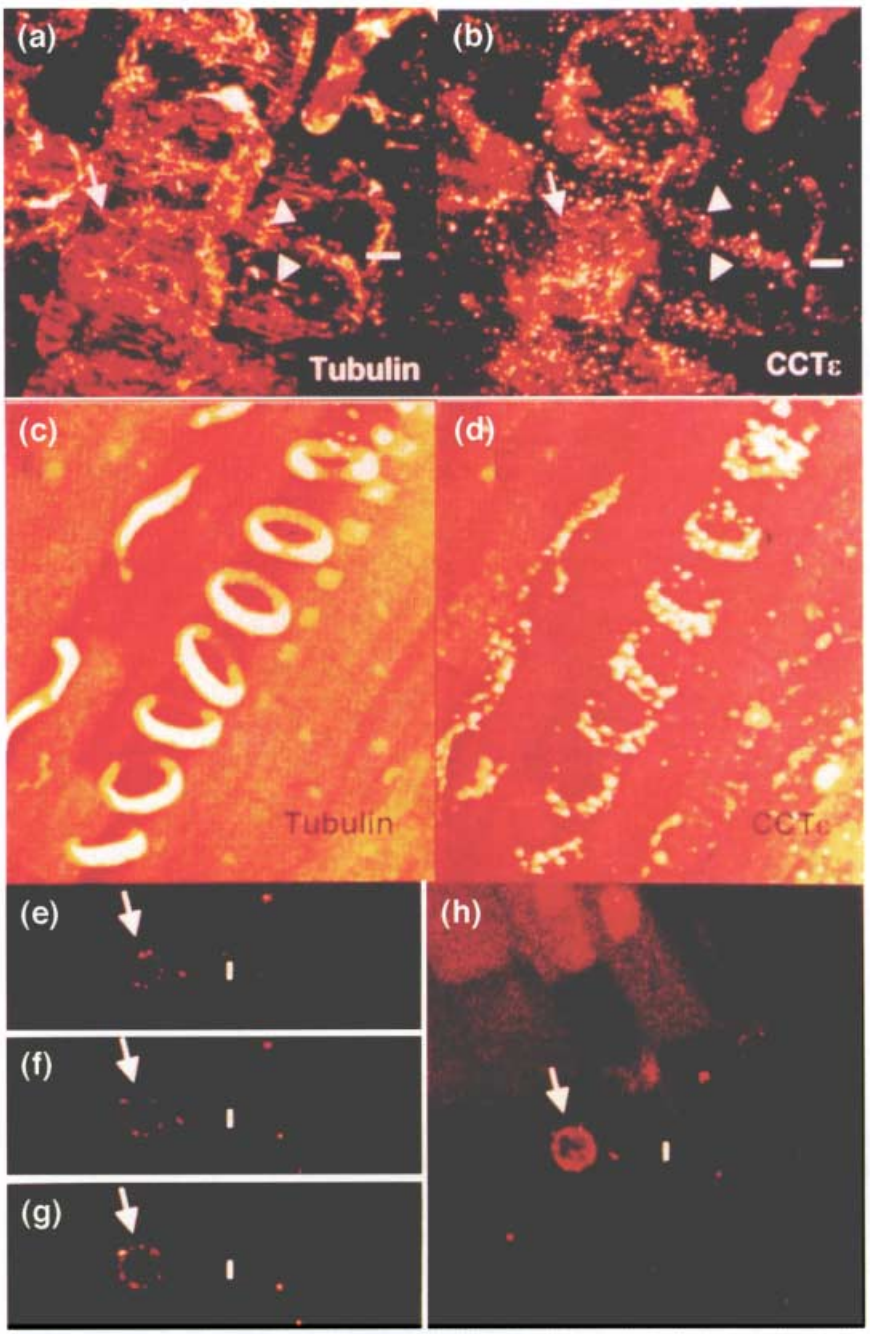

Figure 6. Localization of microtubules and CCTε in different tissues of four day old, etiolated maize coleoptiles.
Double-staining was performed using secondary antibodies labelled with TRITC for detection of tubulin (a, c) and FITC (b, d, e, f) for detection of CCTε. (a, b) Subepidermal cells with transverse cortical microtubules (a). The same cells displaying CCTε (b). The arrows indicate congruent structures, the arrowheads point to discrepancies in the distribution of CCTε and tubulin. (c, d) Sections of young vascular tissues showing protoxylem structures of developing vessels stained with tubulin-antibodies (c) and CCTε-antibodies (d). (e, f and g) Cells of ground parenchyma stained with CCTε primary antibody and a TRITC-conjugated anti-rabbit IgG secondary antibody. The pictures show optical sections of the same nucleus labelled by the CCTε-antibody (arrows). (h) Same nucleus as shown in e, f and g, using the nucleus-specific labelling dye SYTO13 (Molecular Probes). Arrow point to the nucleus. (a, b) Horizontal bars equals 7.2 μm; (e, f, g, h) vertical bars equals 5 μm.

discussion below). The light-dependent increase of CCT-sedimentability is paralleled by a similar behaviour of actin (data not shown), α- and β-tubulin, whereby tubulins tend to increase by age in etiolated coleoptiles (Figure 2a). A far-red-light dependent down-regulation of transcripts encoding different α- and β-tubulins in *Arabidopsis thaliana* have been described by Leu *et al.* (1995) but they presented no data on protein levels. If the mRNA-data from *Arabidopsis* and our data on tubulin in soluble extracts from maize are compatible, one might speculate that there is light-dependent transcriptional control of pools of soluble cytoskeletal components.

Is light-induced sedimentability of CCT-subunits and tubulin correlated to organ growth?

The data shown in Figure 2(a) demonstrate the light-dependent decrease of soluble CCT-subunits and tubulins. Since light induces elongation of the coleoptile, the decrease might be linked to the positive growth response

of this organ. But the light-dependent decrease of CCTε-subunit (Figure 2b) occurs also in the mesocotyl which is growth-arrested in light and elongates in the dark. Obviously, the light-dependent down-regulation of CCTα, CCTε, tubulins and actin is not linked to the growth stage of either the coleoptile and the mesocotyl and therefore is independent from the growth mode (cell division in etiolated mesocotyl and cell elongation in irradiated coleoptiles) as well.

Is the stoichiometry between CCT-subunits controlled by light and development?

Independent of growth, our data suggest light-controlled changes of CCT-subunit composition in soluble extracts of the coleoptile and mesocotyl. In addition, the abundance of CCTε in soluble extracts shows a temporal pattern as it decreases with time. In far-red-treated or etiolated five day old elongating coleoptiles, the amount of soluble CCTε is found to be reduced relative to CCTα (Figure 2a), and the same is observed in the light-grown mesocotyl (Figure 2b). The partial purification of CCT by anion exchange chromatography demonstrated the cofractionation of both CCTα and CCTε in samples from two day old dark- or light-grown coleoptiles suggesting the participation of both subunits in the CCT complex (Figures 3b and c). Since both subunits were detectable in soluble extracts, though in lower amounts after irradiation of the coleoptile (Figure 2a), this result was expected. In extracts from 4.5 day old etiolated coleoptiles, the amount of CCTε decreased stronger than CCTα (Figure 2a), but since both subunits could be still detected (Figure 2a), anion exchange chromatography of the same sample could not reveal a significant quantitative difference between the abundance of CCTα and CCTε (data not shown). Only strong differences as the almost complete disappearance of CCT from soluble extracts prepared from 4.5 day old, far-red-treated coleoptiles became apparent: the Western blot (Figure 3d) displays solely nonspecific signals after prolonged exposure. Therefore, using anion exchange chromatography, no direct evidence for a change in the relation between CCTα and CCTε-subunit content was found.

Differential participation of CCT-subunits in high molecular weight complexes

If the relative abundance between CCT-subunits responds to light and development, this could reflect a differential participation of these subunits in high molecular weight complexes. To test this hypothesis, partial purification by sucrose gradient fractionation, commonly applied to the preparation of CCT, was performed (Frydman *et al.*, 1992; Kubota *et al.*, 1994; Lewis *et al.*, 1992). The results suggest a light-dependent shift of part of CCTα to high molecular weight complexes in young (two day) coleoptiles, whereas CCTε remained in the fractions in which CCT complexes were expected to sediment (Figure 4c). An even more pronounced shift was found for both, α-tubulin and actin that were shifted into fractions where no CCTε, but part of CCTα, was detected (Figure 4c). In contrast, taking extracts from dark-grown, young (two day old) coleoptiles, both CCT subunits and most of the actin and α-tubulin sediment into the same fractions (Figure 4b). This suggests that CCTα and CCTε are part of one CCT particle with actin and α-tubulin bound as substrates. In extracts from old (4.5 day) etiolated coleoptiles, most of the actin and α-tubulin were found in fractions of highest sucrose density. Also, a conspicuous part of both CCT-subunits were detected in high sucrose density fractions (Figure 4d). Interestingly, the immunoprecipitation experiment performed with extracts from old (four day) etiolated coleoptiles (Figure 5) demonstrates that β-tubulin seems to be completely associated with complexes containing CCTε. In contrast, using extracts from far-red-treated, old coleoptiles, no interaction between CCTε and β-tubulin could be detected. This confirms the findings obtained by anion exchange chromatography (Figure 3d) and is consistent with a failure to enrich CCTα and CCTε by sucrose gradient fractionation (data not shown). Our results indicate a shift of CCT-subunit distribution and cytoskeletal components in elongating coleoptiles (Figures 4c and d) and suggest changes in the stoichiometry of CCT depending on the developmental and physiological state of the maize seedlings.

The existence of different types of CCT complexes has been discussed by several authors. Roobol *et al.* (1995) described the existence of a predominantly CCTα-containing particle bound to actin structures in neurites of differentiating nerve cell cultures and Carden and Roobol (1995) assumed that even homo-oligomeric CCTs exist in brain. In murine testis, modifications of CCTϕ subunits (Hynes *et al.*, 1996) as well as a testis-specific subunit have been described ('S6'; Kubota *et al.*, 1994; Kubota *et al.*, 1995a; Kubota *et al.*, 1995b). However, in animals, current studies demonstrate the coordinate mRNA-expression of different subunits during larval stages in nematode (Leroux and Candido, 1995a; Leroux and Candido, 1995b) or during embryonic development of vertebrates (Dunn and Mercola, 1996; Sun *et al.*, 1995). These authors showed by *in situ* hybridization that expression of CCT-transcripts follows a spatio-temporal pattern during embryogenesis and larval development: they demonstrated strong expression in tissues forming the central nervous system and in cell lines dedicated to muscle formation. Physiological studies in *Tetrahymena pyriformis* show upregulation of transcripts encoding CCTγ and CCTη together with β-tubulin mRNA during reciliation (Cyrne *et al.*, 1996; Soares *et al.*, 1994). Similarly, when compared to other organs, strongly enhanced levels of CCTα-mRNA were found in the testis

of adult mice, (Dudley *et al.*, 1984; Gao *et al.*, 1994) and this correlated to the high amounts of tubulins needed during spermiogenesis (Kubota *et al.*, 1994; Kubota *et al.*, 1995a). The data suggest a special requirement for the chaperonin presumably due to enhanced formation of cytoskeleton components. But except in the case of mouse testis, only little is known about the correlation between CCT-mRNA abundance and corresponding protein levels in plants and animals. We found direct correlation between CCTε-mRNA accumulation and protein in developing oat seedlings in special cases only. (Moser *et al.*, submitted). These findings led us to focus on protein analysis for studying the dynamics of CCT composition.

Localization of CCT in maize coleoptiles

Our data show the localization of CCTε at the nuclear surface (Figure 6e, f and g) which has been discussed as a major microtubule-organizing center (MTOC) in plants (Lambert *et al.*, 1993). Even though higher plants lack centrioles, *in vitro* experiments have demonstrated that polymerization of microtubules occurs at the surface of isolated plant nuclei (Mizuno, 1993; Shibaoka and Nagai, 1994; Stoppin *et al.*, 1994). Interestingly, in animal cell cultures, CCT has been shown to be necessary for the proper polymerization of centriolar microtubules (Brown *et al.*, 1996) in a complex together with γ-tubulin (Brown *et al.*, 1996; Moudjou *et al.*, 1996).

Beneath the nuclear envelope, a patchy distribution of CCTε was detected throughout the plant cytosol with only a part of the signal being co-localized to filaments of cMTs indicating that a fraction of CCT is bound to microtubules (Figure 6a, b). A patchy distribution has also been shown for γ-tubulin in non-dividing plant cells (Joshi and Palevitz, 1996). If CCT interacts with γ-tubulin to function as part of MTOCs, such a punctate distribution would be expected. In the case of the strong ring-shaped bundles of microtubules associated with the formation of secondary wall protrusions during early genesis of protoxylem, a (patchy) co-localization of CCTε to microtubular structures is most pronounced (Figure 6c, d). In this case, the associated CCTs possibly deliver folded tubulins that have been newly synthesized.

Does CCT exist in two conformations?

Our studies on the CCT-subunits CCTα and CCTε demonstrated a light- and age-dependency of protein abundance in soluble extracts. The shift of these CCT-subunits, and of their folding substrates, actin and tubulin, into high molecular weight complexes in old (4.5 day) etiolated coleoptiles [a trend already found in young (two day), far-red-treated coleoptiles] suggests an increase in the sedimentability of CCT-subunits. In the extreme case, CCTα and CCTε disappear from soluble extracts in old far-red-treated coleoptiles that have completed elongation. Obviously, light induces a redistribution of CCT-subunits and tubulins, which is not seen when the total pool is analysed (Figure 1a). The question arises if CCT exists in two distinct conformations: one that is bound to pelletable parts of the cytoskeleton and/or membrane fractions, and one that remains soluble. We should also consider whether the extent of solubility is coupled to the function of CCT. CCT has been shown to be bound to membranes (Creutz *et al.*, 1994; Willison *et al.*, 1989) and high molecular weight complexes such as the translational machinery (Frydman *et al.*, 1994; Nimmesgern *et al.*, 1993). By non-denaturing isoelectric focusing, Hynes *et al.* (1995) separated two distinct conformations of CCT that differ in the number of associated proteins. Also, two distinct pools of CCT were found by Hynes *et al.* (1996) during identification of proteins associated to CCT. The light- and development-dependent regulation of CCT-sedimentability in plants suggests soluble and sedimentable forms of CCT with possibly different functions that still have to be elucidated. In future work, biochemical analysis of CCT from these pools, their composition, *in vitro* folding activities and interaction with cofactors should clarify an issue that presumably touches many aspects of plant cytoskeleton organization.

Experimental procedures

Plant material, growth conditions and light sources

Plant material. Maize (*Zea mays* cv. Percival, Asgrow Co., Bruchsal, FRG) was grown at 25°C as described by Nick *et al.*, 1988). Coleoptiles and mesocotyls were immediately frozen in liquid nitrogen after harvest.

Light sources. The following red light sources were applied: far-red (FR) λ_{max} = 740 nm, half-bandwidth = 123 nm, fluence rate = 3.5 W m^{-2} and red (R): λ_{max} = 660 nm, half-bandwidth = 18 nm, fluence rate 6.7 W m^{-2}. Blue light (BL; λ_{max}=436nm, 4.8 W·m^{-2}) was obtained using Phillips 40/18 lamps with a cut off filter at 390 nm. White light (WL) containing UV-A (58 W·m^{-2}) was generated in phytochambers using OSRAM Power Star as a light source.

Preparation of bacterially overexpressed CCTα-protein used for production of anitbody and purification of CCTε-antisera

In order to obtain purified yeast CCTα-subunit, a DNA-fragment encoding the full-size protein was isolated by PCR using genomic yeast DNA as template. The genomic DNA was isolated from yeast spheroblasts using QIAamp extraction Kit (QIAGEN, Hilden FRG). The following primers were synthesized for CCTα-sequence (EMBL M21160) amplification: AACTCGAGTCGACAATGTCCCAATTATTCAATAACTCGCGC (5hefa) and GAGAAAGCTTTCTAGACGCAAAGAATCAATGATCGTGCGG (3hefa). After PCR, the DNA-

fragment of 1690 bp length was digested with *Bam*HI/*Hin*dIII and ligated into the appropriate cloning site of expression vector pQE10 (QIAGEN, Hilden FRG). The construct was used to express a full-size, N-terminal HIS_6-tagged protein that was purified using NTA-agarose following the manufacturer's protocol. (QIAGEN, Hilden FRG). Antisera against CCTα were raised in mice and tried for specificity by cross-reaction tests in Western blots with overexpressed yeast subunits CCTβ, CCTδ, CCTζ and oat CCTε (data not shown). The oat CCTε-antisera from rabbit (Ehmann *et al.*, 1993) was purified against matrice-bound (Eurocell ONB-Carbonat P, Knauer, Berlin FRG) bacterially overexpressed oat-CCTε according to the manufacturers suggestions. For convenience, both the affinity-purified CCTε-antibody as well as the CCTα-antisera from mouse are designated as antibodies in the text.

Protein extraction, anion exchange perfusion chromatography, sucrose gradient fractionation and protein detection by Western blotting

The frozen plant material was homogenized in liquid N_2 using mortar and pestle. For total protein extracts, heated SDS-buffer (130 mM Tris–HCl pH 6.5, 4% SDS (w/v), 10% glycerol (w/v), 10% 2-mercaptoethanol, 8 M urea) was added to the powder and the sample boiled for 10 min with intermittent vortexing . The crude extracts were clarified by centrifugation (15 000 ***g*** for 10 min) and stored at –20°C. Soluble protein extracts were prepared as described by Nick *et al.* (1995), where the authors designate the same preparation as 'cytosolic extract'.

The content of total protein was determined according to Popov *et al.* (1975) with minor modifications: 5–10 µl protein extract were diluted with water to a final volume of 200 µl. 800 µl precipitation solution (90% methanol, 10% acetic-acid, 0.01% amidoblack 10B; SERVA, Heidelberg, FRG) were added and after mixing the suspension was clarified by centrifugation at 18 000 ***g***, 4°C for 20 min. After washing with 1 ml 90% ethanol/10% acetic acid the protein pellet was resolved in 200 µl 0.2 N NaOH. The extinction of the solution was measured at 615 nm.

Anion exchange perfusion chromatography of soluble extracts was performed using a Poros 20HQ column (Perseptive Biosystems, Wiesbaden FRG) fitted into a BioCad pumping system (Perseptive Biosystems). Proteins were fractionated by a salt gradient (0–1.5 M KCl in sample buffer containing 25 mM MES pH 6.9, 5 mM EGTA, 5 mM $MgCl_2$, 1 M glycerol) at a flow rate of 5 ml min^{-1}. Prior to fractionation, extracts were filtrated through a 20 µm filter and 1 mg of protein per sample were loaded onto a 1.66 ml column. Fractions were precipitated with TCA and pellets resuspended in SDS sample buffer. Identical volumes were loaded onto each lane of SDS–PAGE taken for Western blots shown in Figure 3.

Density-gradient fractionation was performed in a linear 10–40% (w/v) sucrose gradient with sucrose dissolved in MES sample buffer. The total volume of the gradient solution was 28 ml in a 36 ml cellulose acetate tube (Kontron, Stuttgart, FRG) that was carefully loaded with 2 ml of soluble extract prior to centrifugation in a swing out rotor (TST28.38, Kontron ,Munich, FRG) at 25 000 ***g*** for 19 h at 4°C. Fractions of 1 ml were collected from top to bottom of the gradient which was determined by measuring the refractive index of each fraction. These were precipitated by TCA and processed as described above.

Polyacrylamide gel electrophoresis (PAGE), staining for proteins, Western blotting and immunodetection was described by Nick *et al.* (1995). The affinity-purified CCTε-antibody (see above) was used at a dilution of 1:500, the antibody against CCTα at 1:300 and the monoclonal antibodies against α- and β-tubulin (Amersham, Braunschweig, FRG) had a working-dilution of 1:300 to 1:500 each.

Co-immunoprecipitation of β-tubulin and CCTε.

Soluble extracts from four day old, etiolated or far-red treated coleoptiles were subjected to immunoprecipitation using a mouse monoclonal antibody against β-tubulin (Amersham, Freiburg, FRG). Aliquots corresponding to 100 µg total protein were supplemented with precipitation buffer (200 mM Tris–HCl pH7.4, 1.5 M NaCl, 2.5% Triton X-100, 0.1% SDS) and 1/10 vol of β-tubulin antibody. The samples were incubated overnight at 4°C with gentle shaking in a overhead tumbler before 1/10 vol of Protein-A–Sepharose was added. The mixture was incubated further at 4°C for 3 h. After sedimentation of Protein-A–Sepharose, the supernatant was removed and stored separate. The sediment was washed five times thoroughly for 5 min with 10-fold diluted precipitation buffer. 100 µl SDS-sample buffer was added to each washed sediment, boiled directly for 5 min and subjected to SDS–PAGE and Western blot together with the supernatants. As a control, the β-tubulin antibody was replaced by mouse normal sera (Sigma, Neu-Ulm, FRG).

Immunolocalization of proteins in maize coleoptiles

Double immune-fluorescence labelling of tubulin and CCTε was performed according to Nick *et al.* (1995) and analysed by confocal laser microscopy (Leitz DM RBE, Leitz GmbH Bensheim, FRG) using an Argon–Crypton laser with 488/546 nm excitation, a 515 nm beam splitter and 515 nm/540 nm emission filters, respectively. Scanning was performed normally in a 8-image linear averaging mode and 1µm-stop intervals. A mixture of monoclonal mouse α- and β-tubulin antibodies (Amersham, Braunschweig, FRG) were diluted 1:100, the CCTε antibody at 1:10 and secondary antibodies at 1:25 working dilution. Negative controls were performed to determine the specifity of signals by replacing one or both primary antibodies by the respective pre-immune or normal sera. The controls confirmed the specifity of the CCTε signal (data not shown), but revealed a strong autofluorescence of cell walls in old xylem tissues that obscured the CCTε-signal (data not shown).

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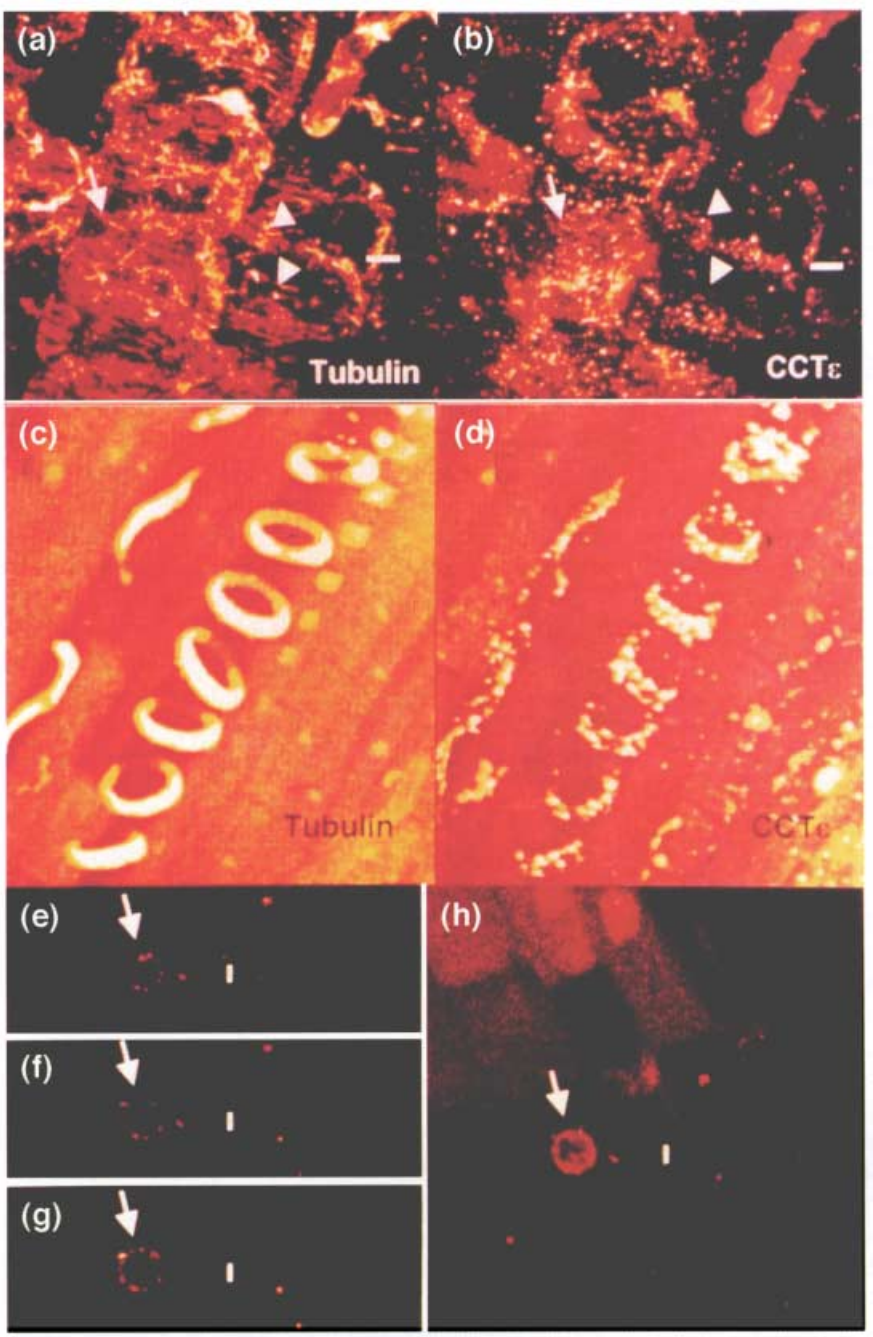
(a)
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