Characterization of Protein and Transcript Levels of the Chaperonin Containing Tailless Complex Protein-1 and Tubulin during Light-Regulated Growth of Oat Seedlings[1]

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In grass seedlings the network of cortical microtubules is reorganized during light-dependent growth of coleoptiles and mesocotyls. We investigated the effects of light-dependent growth on the relative steady-state levels of the mRNAs and protein levels of α-tubulin and the ϵ-subunit of the chaperonin containing tailless complex protein-1 in oat (*Avena sativa*) coleoptiles, which were grown in different light conditions to establish different growth responses. The soluble pools of the ϵ-subunit of the chaperonin containing tailless complex protein-1 and α-tubulin decreased in nonelongating coleoptiles, suggesting that the dynamics of the light-regulated soluble pool reflect the processes occurring during reorganization of cortical microtubules. The shifts in pool sizes are discussed in relation to the machinery that controls the dynamic structure of cortical microtubules in plant cells.

The physiological adaptation of plants to different environmental factors is reflected in the level of cell organization, the structure of the cytoskeleton, and hence the cortical microtubules. This has been shown for light and gravistimuli (Nick et al., 1990), endogenous signals such as hormones (Shibaoka, 1991), and combinations of light and hormones (Iwata and Hogetsu, 1989b; Zandomeni and Schopfer, 1993; Nick and Schäfer 1994; Toyomasu et al., 1994). It is assumed that the orientation of microtubules influences the incorporation of cell wall microfibrils and hence the shape of plant cells (Wymer and Lloyd, 1996). Elongating cells often contain transverse-oriented cortical microtubules, whereas growth arrested cells contain longitudinally orientated cortical microtubules (Iwata and Hogetsu, 1988). This light-dependent reorientation of cortical microtubules would require destabilizing existing microtubules and polymerizing new microtubules, often in an orientation perpendicular to the growth direction of the plant cell. These processes should include changing pool sizes of free to polymerized tubulin, the need for de novo synthesis of tubulins, and the presence of microtubule organizing centers.

The cytosolic chaperonin containing tailless complex protein-1 (CCT) and several cofactors are characterized as the folding machinery for the cytoskeletal proteins actin, and α- and β-tubulins in vivo and in vitro (Lewis et al., 1992; Yaffe et al., 1992; Sternlicht et al., 1993). CCT is a heterooligomeric molecular chaperone complex containing eight different, but related polypeptides. Mutations in yeast genes of different CCT subunits affect microtubule-mediated processes such as cell division and distribution of nuclei (Ursic and Culbertson, 1991; Chen et al., 1994; Miklos et al., 1994; Ursic et al., 1994; Vinh and Drubin, 1994). In animals the coordinated expression of CCT subunits and tubulins has been found in the testis of rodents where CCT up-regulation during spermatogenesis meets the requirement for production of high amounts of tubulins needed in this tissue (Silver et al., 1987; Kubota et al., 1994, 1995). In the protozoan ciliate *Tetrahymena,* reciliation processes after shock treatment include a strong coordinate accumulation of tubulin and CCT transcripts (Soares et al., 1994).

Further studies demonstrate the simultaneous mRNA accumulation for different CCT subunits during larval stages in nematodes (Leroux and Candido, 1995a, 1995b). During embryonic development of vertebrates, high expression levels of vertebrate CCT mRNA are predominantly found in tubulin-rich tissues forming the central nervous system or in cell lines dedicated to muscle formation (Sun et al., 1995; Dunn and Mercola, 1996). The central function of CCT for proper organization of the microtubular network is supported by studies showing that CCT acts as part of the microtubular organizing center in animal systems (Brown et al., 1996). CCT can also be detected in axons of growing neurites away from the site of protein synthesis of the cell (Roobol et al., 1995) and different CCT subunits behave as microtubule-

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associated proteins in vitro (Roobol et al., 1999). Therefore CCT might not function solely as a molecular chaperone for newly synthesized proteins, but might also be involved in cytoskeletal assembly. The observations that tubulins can be kept in solution by cofactors of CCT (Tian et al., 1996) and maintained in an activated conformational state (Tian et al., 1997; Vainberg et al., 1998) suggest that the CCT system is able to manage variable pool sizes of tubulins, which suggests further functions of CCT and cofactors beyond folding of proteins. Thus CCT may also play an important role in microtubule reorientation in plant cells.

Using oat (*Avena sativa* cv Victory) seedlings that pass through different growth stages, we investigated the relationship between tubulin mRNA synthesis and the corresponding protein, as well as the concomitant presence of CCT chaperonin, to elucidate the coregulation of CCT and its substrates as found in animal systems. We found a light-dependent decrease in the soluble pools of tubulin and CCT in oat coleoptiles, which ceased growth after treatment with light. The total amounts of tubulins and CCT were not affected by light treatments.

RESULTS

CCTϵ Is Part of a High-M_r Complex in Oat

Partial purification of CCT from whole oat seedlings revealed CCTϵ to be part of a high-M_r complex (Fig. 1). CCTϵ, as revealed by immunoblot analysis, sedimented into fractions containing 19% to 21% Suc (Fig. 1B). Silver staining of the partially purified complex separated on SDS-PAGE revealed bands of 50 to 70 kD (Fig. 1C) resembling the polypeptide pattern from similar purification schemes for mammalian CCT (Frydman et al., 1992; Lewis et al., 1992; Kubota et al., 1994). The western blot of the purified complex using the anti-CCTϵ antibody and a prestained M_r marker detected one prominent band with an apparent molecular mass of 65 kD (Fig. 1D). When unstained markers were used, CCTϵ was found at 60 kD apparent molecular mass, corresponding to the calculated molecular mass for oat CCTϵ of 59 kD (Ehmann et al., 1993).

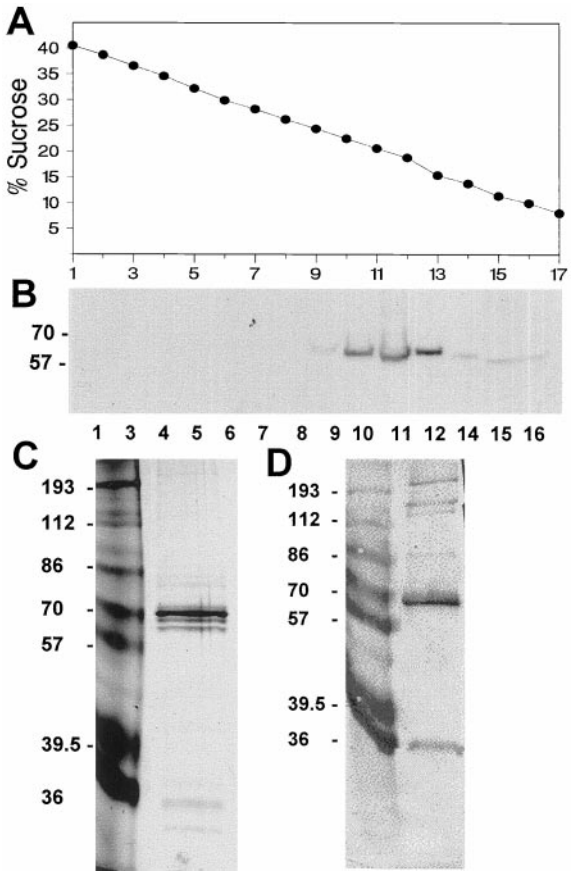


Figure 1. Partial purification of the CCT complex from whole oat seedlings. Soluble extracts of dark-grown oat seedlings were fractionated on a 10% to 40% (w/v) Suc gradient. A, Suc densities are shown as the percentage of Suc per fraction. B, The CCTϵ-subunit was found to sediment at 20% (w/v) Suc as determined by western-blot analysis of gradient fractions probed with the anti-CCTϵ-specific antiserum. C and D, Analysis on a 10% (w/v) SDS-polyacrylamide gel of purified CCT complex. C, SDS-PAGE and silver staining of the purified CCT complex. D, Western-blot analysis of purified CCT complex probed with anti-CCTϵ antiserum. Protein M_r markers with their respective molecular masses in kD are shown on the left.

Light Control of Soluble Pools of CCTϵ and α-Tubulin

We used dark-grown seedlings exposed to different light qualities to test the hypothesis that the distribution of CCT within the cell may depend on the growth status of the organ. Previous experiments with etiolated seedlings suggested that there were no significant changes in the total amount of CCTϵ, and α-and β-tubulin in response to light compared with the light-dependent down-regulation of type A phytochrome and induction of light harvesting complex II (LHCII; data not shown). In addition there were no significant changes in the total amount of CCTϵ, and α- and β-tubulin in response to growth of the coleoptile or mesocotyl (data not shown). These experiments did not address changes in specific pools of these proteins. Therefore, soluble extracts from coleoptiles were prepared and examined for CCTϵ and α-tubulin abundance. The abundance of CCTϵ and α-tubulin in coleoptiles decreased after irradiation (Fig. 2, B and C, respectively; western blots shown as insets). All light treatments decreased the growth rate of coleoptiles compared with dark-grown seedlings, and growth ceased after 24 h (data not shown). Growth inhibition of coleoptiles was accompanied by the emergence of the primary leaf. In contrast, in soluble extracts from etiolated seedlings at the beginning of their elongation phase, the amount of CCTϵ and α-tubulin increased between 4 and 5 d after sowing (Fig. 2, A and B, compare D and D + 24 h values), suggesting a positive correlation between

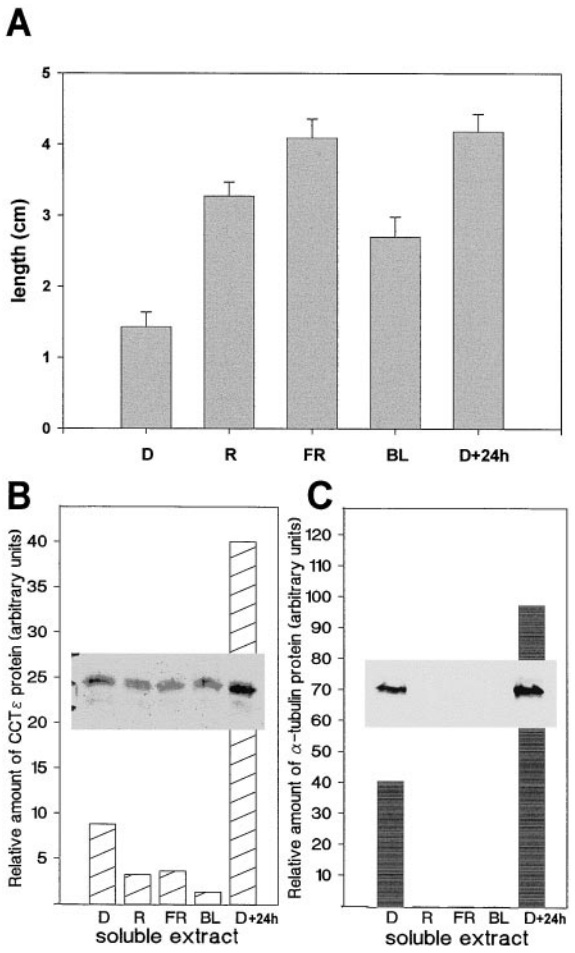


Figure 2. Quantification of western blots using soluble extracts from etiolated seedlings grown for 4 d in the dark before transfer to red (R), far-red (FR), or blue light (BL) for 24 h. Dark-grown controls were taken at onset of irradiation (D) and at the end of irradiation (D + 24 h). A, Coleoptile length of etiolated or irradiated seedlings. In contrast to etiolated coleoptiles, the coleoptiles of the light-grown plants completed elongation. B, Relative amounts of soluble fraction of CCTϵ protein. C, Relative amounts of soluble fraction of α-tubulin.

growth and the presence of soluble forms of CCTϵ and α-tubulin .

Regulation of CCTϵ and α-Tubulin in Coleoptiles of Seedlings Grown in Continuous Light

Oat seedlings grown in continuous light without a preceding dark period were analyzed to confirm data on soluble pools of CCT and tubulin (Fig. 3). These plants have very short mesocotyls and therefore display a very different phenotype than etiolated seedlings. The irradiated coleoptiles had stopped growing at the time of harvest and primary leaves had emerged, in contrast to etiolated coleoptiles, which were shorter and just beginning their elongation phase (Figs. 3A and 2A). Blue light decreased the total amount of CCTϵ and α-tubulin to about 50% when compared with dark-grown seedlings (Fig. 3, B and C; left panels labeled "total extract"). The total amount of α-tubulin also decreased by 25% to 30% in red light and far-red light (Fig. 3C, left panel). In soluble extracts, red light and blue light induced a strong decrease of the amount of CCTϵ and particularly of α-tubulin (Fig. 3, B and C, respectively, right panels labeled "soluble extract") compared with dark control. Blue light was most effective in decreasing the amount of CCTϵ and α-tubulin , whereas in far-red light, levels of soluble α-tubulin and CCTϵ remained high. The corresponding transcript analysis (Fig. 3D) indicated a light-dependent down-regulation of CCTϵ and α-tubulin mRNA of seedlings kept in red light, far-red light, and blue light.

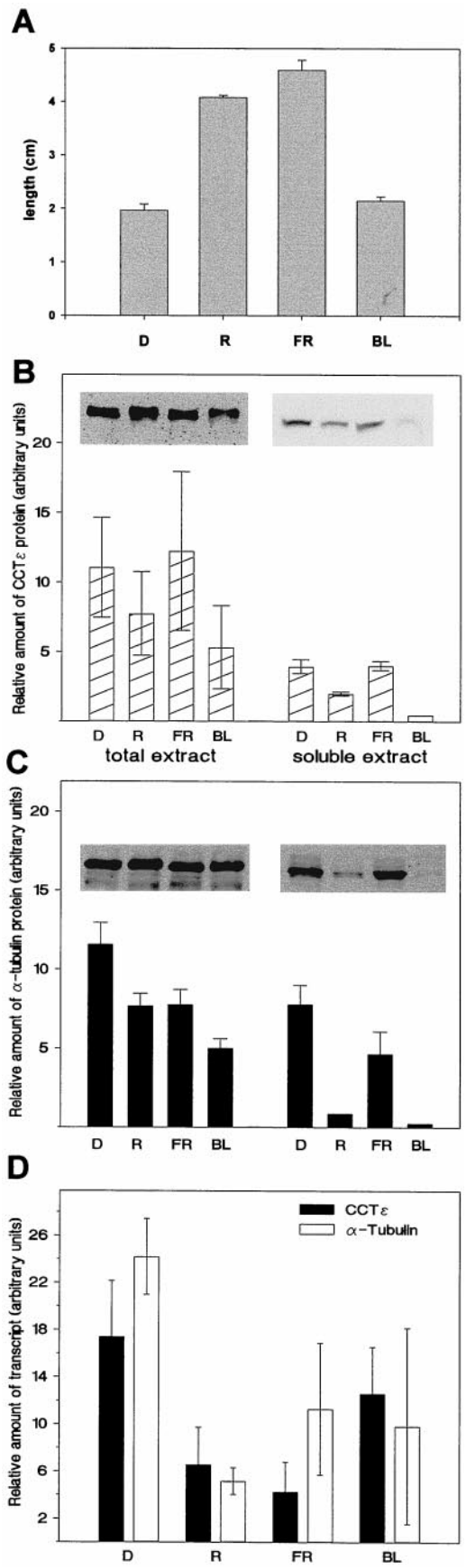
DISCUSSION

We purified CCT from oat as a high-M_r complex with CCTϵ as one of several subunits (Fig. 1). The polypeptide patterns for the CCT complex of oat and cucumber seedlings (Ahnert et al., 1996) are similar to mammalian CCT (Frydman et al., 1992; Lewis et al., 1992; Kubota et al., 1994). Therefore it seems likely that plants also have a standard eukaryotic CCT machinery for the folding of cytoskeletal proteins. We also observed a 30-kD proteolytic fragment of CCTϵ. Similar sized proteolytic fragments were detected during biochemical extraction of CCT from mouse testis and can be assigned to a distinct conformational state of the CCT complex, which differs in the number of bound proteins and the susceptibility to proteolysis (Hynes et al., 1995).

The present analysis was done to obtain insights into the in vivo function of CCT based on the hypothesis that the dynamics of plant microtubules in growing cells imply the need for newly synthesized tubulins and factors involved in organizing the microtubular network (Himmelspach et al., 1997).

Plant Growth Responses and Control of Tubulin Abundance

Light-dependent growth inhibition of seedling hypocotyls with the parallel decrease of β-tubulin transcripts has been described in white lupine (Vassilevskaia et al., 1996) and Arabidopsis (Leu et al., 1995). Leu et al. (1995) reported a phytochrome-dependent decrease of Arabidopsis α- and β-tubulin transcripts, analyzing transcript-abundancies of six α-tubulin and nine β-tubulin mRNAs. Similarly, phytochrome-controlled down-regulation of β-tubulin mRNA may be linked to the light-dependent growth arrest of oat coleoptiles (Colbert et al., 1990). Mendu and Silflow (1993a) describe a positive correlation between hormone-induced oat internode elongation, and α- and β-tubulin transcript levels. Thus the transcriptional control of the amount of tubulins may be tightly linked to the growth state of the plant tissues.



Our analysis of α-tubulin transcript levels confirmed down-regulation in light-treated oat coleoptiles (Fig. 3D). The levels of total CCTϵ, and α- and β-tubulin protein did not correlate with the light-dependent changes of steady-state transcript levels (Fig. 3 and data not shown). Similarly, a strong down-regulation of β-tubulin transcripts with no significant change of total pools of tubulin protein has been described in soybean internodes (Bustos et al., 1989). Because both α- and β-tubulin should be present in equimolar amounts to dimerize prior to tubulin formation, translational and post-translational control is required to compensate for the different expression characteristics of tubulin transcripts.

The limitation of our approach is the measurement of total tubulin transcript and protein levels. The tubulins are known to be encoded by families of related genes. In maize the individual tubulin isotypes exhibit tissue-specific patterns of expression (Joyce et al., 1992; Villemur et al., 1994). Mendu et al. (1993b) obtained evidence for a minimum of eight β-tubulin genes in oat, and it is likely that tubulins in oat also show patterns of differential transcript accumulation and isotype expression. In Arabidopsis only two of the nine β-tubulin genes are strongly down-regulated by light (Leu et al., 1995). Therefore it is clearly important to establish which members of the tubulin gene family of oat account for the light-induced down-regulation. This issue can only be addressed using gene-specific probes or isotype-specific antibodies for oat tubulins. The unavailability of these tools may be the reason that the changes we observed in total transcript and protein abundance of tubulin were very small compared with the changes in strongly light-regulated polypeptides such as phytochrome A and LHCII (data not shown).

Coordinate Light Regulation of CCTϵ and α-Tubulin Abundance in Soluble Extracts from Oat Coleoptiles

In soluble protein extracts we demonstrated a concurrent red light- and blue light-dependent decrease of both CCTϵ and α-tubulin. These results were obtained for seedlings grown for 4 d in the dark prior to a 24-h light treatment (Fig. 2), as well as in plants grown for 4 d in continuous light (Fig. 3). All of the

Figure 3. Light control of soluble pools CCTϵ and α-tubulin in oat coleoptiles. A, Growth of etiolated or irradiated coleoptiles. Note that the coleoptiles of light-grown plants (red light [R], far-red light [FR], and blue light [BL]) completed elongation, whereas etiolated coleoptiles (D) were at the beginning of the elongation phase (compare with Fig. 2A). B, Western-blot analysis and quantification of CCTϵ protein in total (left one-half of graph) and soluble extracts (right one-half of graph); C, same as B, but detection of α-tubulins. Values represent means from at least three independent experiments. D, Transcript accumulation of CCTϵ and α-tubulin in the coleoptile of 4-d-old oat seedlings grown in dark (D) or irradiated with red light (R), far-red light (FR), or blue light (BL). Values represent the mean of four independent measurements.

light treatments induced growth arrest of the coleoptiles. The growth inhibition of coleoptiles in seedlings grown in continuous light was paralleled by a decrease of α-tubulin and CCTε mRNA levels (Fig. 3C), whereas the amount of soluble CCTε and α-tubulin increased in the elongating coleoptiles of etiolated plants. These results indicate that growing tissues accumulate soluble α-tubulin and CCTε. Oat coleoptiles grown in continuous far-red light showed only a small decrease in soluble CCTε and α-tubulin levels (Fig. 3, B and C), whereas soluble pools of CCT subunits, tubulins, and actin are strongly downregulated in far-red-light-grown maize seedlings (Himmelspach et al., 1997). This light-induced sedimentability of CCT subunits and tubulin is independent of the growth stage of the maize coleoptile.

Since soluble pools of CCTε and α-tubulin decreased in nonelongating oat coleoptiles, the distinct structural arrangement of cortical microtubules in the oat coleoptile (Iwata and Hogetsu, 1988, 1989a) may be reflected on the biochemical level by different solubilities of tubulins. Growing tissues that contain transverse-oriented cortical microtubules may be enriched in soluble tubulin compared with growth-arrested cells possessing longitudinally orientated microtubules and lower amounts of soluble tubulin.

Our current hypothesis is that in plants the chaperonin itself is neither controlled by light nor coleoptile elongation. Rather, because of the central function of CCT in the folding of tubulins, chaperonin levels may be adjusted to the total pool sizes of tubulins and therefore be controlled by the actual amount of substrate present in the cytosol.

CONCLUSION

The dynamics of tubulin and CCT abundance in soluble extracts might reflect structural reorganization of the microtubular network of the plant cytoskeleton during light-dependent growth responses. It is still an open question as to what extent the soluble pools contribute to the recruitment of tubulins during light-dependent reorganization of microtubules and concomitant growth responses. It is obvious that CCT redistributes with tubulins in a similar manner, supporting the hypothesis that CCT is involved in reorganization of plant microtubular networks.

The results presented here demonstrate that the mechanisms regulating cytoskeletal components during growth responses are complex, involving both transcriptional and post-translational control. A fraction of the cytoskeletal proteins remained soluble in the plant cell in a light- and growth-dependent manner. Elongating coleoptiles maintained high levels of soluble tubulins. These changes in levels of solubility might be controlled by the CCT system and its cofactors. Therefore CCT may be one important checkpoint for controlling the flux of newly formed tubulins and probably manages the soluble pools of tubulins.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Light Sources

Oat (*Avena sativa* cv Victory) seedlings were grown on moist vermiculite at 25°C under the light conditions described below. Coleoptiles were harvested under safe green light. The following light wavelengths were used: far-red: λ_{max} = 740 nm, one-half-bandwidth = 123 nm, fluence rate = 3.5 W m^{-2}, red: λ_{max} = 660 nm, one-half-bandwidth = 18 nm, fluence rate 6.7 W m^{-2}. Blue light, λ_{max} = 436 nm, fluence rate = 4.8 W m^{-2}, was obtained using a fluorescent tube (TL 40/18, Philips, Hamburg, Germany) behind a 390-nm cutoff filter. Safe green light, λ_{max} = 525 nm, one-half-bandwidth = 21 nm, fluence rate 0.3 W m^{-2}, was used for harvesting. The length of coleoptiles was measured to the nearest millimeter with a ruler.

RNA Extraction and Northern Analysis

Total RNA extraction and northern blots were prepared as described by Ehmann et al. (1991). The following DNA probes were employed for mRNA-detection:

A cDNA-fragment encoding the full-length of the CCTε polypeptide from oat (Ehmann et al., 1993; ASTCP-K19, EMBL no. X75777).

A cDNA-fragment for α-tubulin from oat containing the full reading frame (EMBL no. X97446) that had been obtained by reverse transcriptase-PCR (Kawasaki, 1990) using total RNA from oat seedlings. The fragment was amplified using a 5′ primer with the sequence 5′-GAGAGAGCTCGCATGCAAGAGAGATCATCAGCATCC-3′ and a 3′ primer with the sequence 5′-AGGTACCAGAGAACTACTTATTAACC-3′.

A cDNA-fragment for β-tubulin from oat containing the full open reading frame (EMBL no. X54852), which was obtained by reverse transcriptase-PCR as mentioned above. The fragment was amplified using a 5′ primer with the sequence 5′-GAGAGAGCTCGCATGCGAGAGATCCT-(GC)CACATCCA(AG)GGC-3′ and a 3′ primer with the sequence 5′-GAGAAAGCTTGGATCTCCTTACATGTCCTCAGCCTGC-3′.

For northern analysis the RNA was transferred onto a nylon membrane (Roche Diagnostics, Mannheim, Germany) optimized for non-radioactive detection. Prehybridization and hybridization of northern blots with digoxygenin-labeled probes were performed following the protocol for Southern blots supplied by Roche Diagnostics using a 50% (v/v) formamide hybridization buffer. Membranes were washed twice in 2× SSC/0.2% (w/v) SDS at 42°C for 5 min, and once with 2× SSC/0.2% (w/v) SDS at 58°C and 1× SSC/0.2% (w/v) SDS at 60°C. After hybridization, visualization of mRNAs was achieved by exposing a x-ray film (Fuji Photo Film, Tokyo) to the nylon-membranes, with disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2-(5-chloro)tricyclo(3.3.1.1^{3,7})decane]-4-yl) phenyl phosphate as a chemiluminescent agent following the manufacturer's manual (Roche Diagnostics).

Protein Extraction and Western Blotting

The frozen plant material was ground in liquid nitrogen using a mortar and pestle. For total protein extracts, heated SDS-buffer (130 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 6.5, 4% [w/v] SDS, 10% [w/v] glycerol, and 10% [v/v] 2-mercaptoethanol) was added to the powder and the sample was 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). Total protein amounts were determined according to a modified method after Popov et al. (1975): 5 to 10 μL of protein extract were diluted with water to a final volume of 200 μL, and 800 μL of precipitation solution (90% [v/v] methanol, 10% [v/v] acetic-acid, and 0.01% [w/v] amidoblack 10B; Serva, Heidelberg, Germany) was added. After mixing, the suspension was clarified by centrifugation at 18,000*g* at 25°C for 20 min. After washing with 1 mL of 90% (v/v) ethanol-10% (v/v) acetic-acid, the protein pellet was dissolved in 1 mL of 0.2 N NaOH. The extinction of the solution was measured at 615 nm.

Twenty micrograms of total protein per lane was separated by SDS-PAGE according to the method of Laemmli (1970). Gels were stained with 0.2% (w/v) Coomassie Blue R-750, 7% (v/v) acetic acid, and 50% (v/v) methanol and destained with 7% (v/v) acetic acid and 20% (v/v) methanol. For immunoblots, after SDS-PAGE, proteins were transferred to nitrocellulose filters (0.2 μm, BA 85, Schleicher & Schuell, Keene, NH) in a semidry chamber. After 1 h of incubation with Tris-buffered saline plus Tween 20 (TBST; 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.02% Tween 20) supplemented with 5% non-fat milk powder, the nitrocellulose filters were incubated for 1 h with the antibodies indicated below, washed five times for 5 min each with TBST, incubated with peroxidase-coupled secondary antibody (diluted 1:3,000 in TBST) for 1 h, and washed five times for 5 min each with TBST. The blots were developed using the enhanced chemiluminescence western-blotting detection system (Amersham, Braunschweig, Germany) according to the manufacturer's protocol. Polyclonal antisera raised against CCTε-protein was described by Ehmann et al. (1993); monoclonal antibodies for detection of α- and β-tubulin were purchased from Amersham (N356 and N357, Amersham). Polyclonal antisera raised against LHCII from white mustard (Harter et al., 1993) and type A phytochrome (pAVR, Mummert et al., 1993) were used for the detection of strongly light-controlled polypeptides. Equal protein loading and integrity of protein samples were verified on Coomassie Blue-stained gels in parallel to each western blot or by Ponceau S red staining of the blot membrane.

Partial Purification of CCT

Four-day-old dark-grown oat seedlings were harvested and homogenized in 0.8 volume of extraction buffer (0.1 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-NaOH, pH 7.5, 0.2 M Suc, 0.15 M NaCl, 5 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 μg/mL antipain, and 1 μg/mL leupetin). The homogenate was filtered through two layers of miracloth and centrifuged for 1 h at 100,000*g*. The supernatant was applied to an anion-exchange column (Poros HQ50, PerSeptive Biosystems, Framingham, MA) equilibrated in buffer A (50 mM HEPES-NaOH, pH 7.5, 0.1 M NaCl, 10% [w/v] glycerol, and 1 mM dithiothreitol [DTT]). Bound proteins were eluted using a linear gradient 0.1 to 0.5 M NaCl. The eluate was analyzed by western blotting using anti-CCTε antisera to determine the peak fractions of CCT. CCTε-containing fractions were pooled, desalted by ultrafiltration (100-kD filter, Millipore, Bedford, MA), and applied onto a second anion-exchange column (MonoQ, Amersham Pharmacia Biotech, Freiburg, Germany) equilibrated in buffer B (20 mM Tris-HCl, pH 8.0, 10% [w/v] glycerol, and 1 mM DTT), and bound proteins were eluted with a linear gradient of 0 to 1 M $MgCl_2$. CCT-containing fractions were pooled, concentrated by ultrafiltration, and separated on a 10% to 40% (w/v) continuous Suc gradient in buffer C (50 mM HEPES-NaOH, pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, and 1 mM DTT) at 26,000 rpm for 18 h at 4°C (Tst 28.38 swing out rotor, Kontron, Neufarn, Germany). The gradient was fractionated into fractions of 2 mL from bottom to top.

Quantification of Northern and Western Blots

Exposed x-ray films (Fuji Photo Film) from chemiluminescent western or northern blots were scanned on a JX330 (Panasonic, Tokyo). Stored image files were analyzed using ONE-Dscan 1.0 Software (Scanalytics, Fairfax, VA). The data represent the mean from two to three parallel experiments unless indicated otherwise in the figure legend. Error bars were calculated as averages of SD.

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