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Regular paper

Chlorophyllide *a* oxygenase mRNA and protein levels correlate with the chlorophyll *a*/*b* ratio in *Arabidopsis thaliana*

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Abstract

Plants can change the size of their light harvesting complexes in response to growth at different light intensities. Although these changes are small compared to those observed in algae, their conservation in many plant species suggest they play an important role in photoacclimation. A polyclonal antibody to the C-terminus of the *Arabidopsis thaliana* chlorophyllide *a* oxygenase (CAO) protein was used to determine if CAO protein levels change under three conditions which perturb chlorophyll levels. These conditions were: (1) transfer to shaded light intensity; (2) limited chlorophyll synthesis, and (3) during photoinhibition. Transfer of wild-type plants from moderate to shaded light intensity resulted in a slight reduction in the Chl *a*/*b* ratio, and increases in both *CAO* and *Lhcb1* mRNA levels as well as CAO protein levels. CAO protein levels were also measured in the *cch1* mutant, a P642L missense mutation in the H subunit of Mg-chelatase. This mutant has reduced total Chl levels and an increased Chl *a*/*b* ratio when transferred to moderate light intensity. After transfer to moderate light intensity, *CAO* mRNA levels decreased in the *cch1* mutant, and a concomitant decrease in CAO protein levels was also observed. Measurements of tetrapyrrole intermediates suggested that decreased Chl synthesis in the *cch1* mutant was not a result of increased feedback inhibition at higher light intensity. When wild-type plants were exposed to photoinhibitory light intensity for 3 h, total Chl levels decreased and both *CAO* mRNA and CAO protein levels were also reduced. These results indicate that CAO protein levels correlate with *CAO* mRNA levels, and suggest that changes in Chl *b* levels in vascular plants, are regulated, in part, at the *CAO* mRNA level.

Abbreviations: CAO – chlorophyllide *a* oxygenase; *cch1* – conditional chlorina 1; Chl – chlorophyll; DMF – N,N,-dimethyl formamide; Elip – early light inducible protein; h – hours; LHC – light harvesting complex; Lhcb – light harvesting complex II protein; PChlide – protochlorophyllide; PPIX – protoporphyrin IX; PS – photosystem; WT – wild type

Introduction

In higher plants and green algae chlorophyll (Chl) *a* is bound to the reaction center proteins and the internal antennae of PS I and PS II while both Chl *a* and Chl *b* are bound to the outer light-harvesting complexes of PS I and PS II, termed LHCI and LHCII. Structurally, Chl *a* and Chl *b* only differ in the side chain

at position C-7, with a methyl group being present in Chl *a* and a formyl group being present in Chl *b*. Chlorophyllide *a* oxygenase (CAO) catalyzes the oxygenation of Chlide *a* producing Chlide *b* (reviewed in Rüdiger 2002). The *CAO* gene was first isolated in *Chlamydomonas* (Tanaka et al. 1998), and mutation in a homologous gene was shown to result in complete Chl *b* deficiency in *Arabidopsis thaliana* (Espineda et al. 1999). The *CAO* gene appears to be conserved in Chl *b* containing organisms suggesting that it evolved once in a progenitor of oxygenic photosynthetic bacteria (Tomitani et al. 1999). Recombinant CAO has been shown to convert Chlide *a* into Chlide *b* (Oster et al. 2000) most likely by a two-step oxygenation, and then the enzyme Chl synthase adds a hydrophobic phytol tail (Oster et al. 1997) producing Chl *a* and Chl *b*, respectively.

The regulation of Chl *a versus* Chl *b* synthesis has been a long-standing question in tetrapyrrole research. In the algae *Dunaliella tertiolecta*, the amount of LHCII changes dramatically at different light intensities (Sukenik et al. 1990). Transfer of *Dunaliella salina* from high light intensity to low light intensity results in 3-fold increase in the levels of total Chl and a 75% reduction in the ratio of Chl *a* to Chl *b* within 24 h. The reduction in the Chl *a*/*b* ratio is a result of increased synthesis of Chl *b* and production of a larger light-harvesting complex. This increase in the synthesis of Chl *b* is accompanied by a rapid (1.5 h) 5-fold induction of *CAO* mRNA levels (Masuda et al. 2002, 2003). Thus in *Dunaliella*, it is evident that regulation of *CAO* gene expression is an important step in increasing Chl *b* synthesis.

In plants, the size of the light-harvesting complexes can change, but the changes are not as dramatic and significantly slower than those observed in *Dunaliella*. In pea, the Chl *a*/*b* ratio decreased from 3.2 for plants grown at 840 μmol photons $m^{-2}\,s^{-1}$ to 2.3 for plants grown at 42 μmol photons $m^{-2}\,s^{-1}$, a 28% decrease (Leong and Anderson 1984; Larsson et al. 1987). Acclimation from 400 to 100 μmol photons $m^{-2}\,s^{-1}$ was measured in *A. thaliana* (Walters and Horton 1994), and it was found that the Chl *a*/*b* ratio decreased 16% after 3 days of exposure to the lower light intensity. Whether the regulation of CAO levels plays a role in regulating antenna size in plants is not known, but a 2- to 3-fold overexpression of the *CAO* gene in *A. thaliana* did result in a 10% increase in Chl *b* and LHCII levels (Tanaka et al. 2001), suggesting that increased *CAO* mRNA levels can affect the size of LHCII. In this paper, we grow *Arabidopsis* under different light intensities to determine whether changes in the Chl *a*/*b* ratio correlate to changes in the levels of endogenous *CAO* mRNA and protein.

Along with increases in *CAO* mRNA, an increase in *Lhcb* gene expression has been observed when *D. salina* was transferred from high to low light intensity. *Lhcb* encodes the major light-harvesting proteins of PS II, and binds the majority of Chl *b* in plants, and thus it is not surprising that these two genes would be coregulated (Masuda et al. 2002). Also, the enhancement of Chl *b* synthesis in a CAO^{+} strain of *Synechocystis* PCC6803 by the introduction of a pea *Lhcb* gene suggests that these two proteins interact in some manner that promotes Chl *b* synthesis (Satoh et al. 2001; Xu et al. 2001). Increased *Lhcb* mRNA levels were also observed one day after transfer from 400 to 100 μmol photons $m^{-2}\,s^{-1}$ in *A. thaliana* (Walters and Horton 1994), although the increased levels of *Lhcb* mRNA were not maintained upon longer exposure to the lower light intensity. A 90% decrease in *Lhcb* mRNA levels was observed when barley plants were subject to 1–3 days of light stress (1500 μmol photons $m^{-2}\,s^{-1}$), however after 8 days at this light intensity, the Chl *a*/*b* ratio only increased 9% (Montané et al. 1998). Furthermore, antisense inhibition of *Lhcb* gene expression in tobacco to nearly undetectable levels did not result in any changes in the total amount of Chl or the Chl *a*/*b* ratio (Flachmann and Kühlbrandt 1995). Recently, *Lhcb1* and *Lhcb2* mRNAs were reduced to undetectable levels in antisense *Arabidopsis* plants, and these plants had no detectable Lhcb1 or Lhcb2 protein, and had reduced Chl levels as well as an increased Chl *a*/*b* ratio (Andersson et al. 2003). Thus in plants a correlation between *Lhcb* mRNA levels and Chl *a*/*b* ratios has not been apparent, unless the mRNAs are completely absent.

A number of Chl *b*-deficient mutants have been isolated in many plant species. Many of these lines show a light-intensity dependent loss of Chl. Under conditions of limited Chl availability, the regulation of Chl *a versus* Chl *b* synthesis is critical, and the production of Chl *a* bound to reaction centers is favored over the production of Chl *b* bound to peripheral light-harvesting complexes (Droppa et al. 1988; Greene et al. 1988; Knoetzel and Simpson 1991). Feeding the tetrapyrrole precursor, aminolevulinate, to many different Chl *b*-deficient mutants in the dark led to accumulation of protoporphyrin IX, suggesting that the enzyme Mg-chelatase was defective in these lines

(Falbel and Staehelin 1996). A Chl *b*-deficient mutant was isolated in *A. thaliana*, and it was named conditional chlorina 1, *cch1*, due to its light-intensity dependent phenotype. This line was found to harbor a P642L mutation in the *ChlH* gene encoding the H subunit of Mg-chelatase (Mochizuki et al. 2001), confirming the role of this enzyme in the Chl *b*-deficient phenotype. Here, the *cch1* mutant is used because it shows a significant loss of Chl *b* when transferred to moderate light allowing us to ask whether *in vivo* loss of Chl *b* correlates to *CAO* mRNA and protein levels. In addition, the cause of reduced Chl synthesis at moderate light in the *cch1* mutant was explored. Flux through the tetrapyrrole pathway was measured to determine whether inhibition of Chl synthesis was increased when the *cch1* mutant was transferred to moderate light intensity.

Materials and methods

Plant material and growth conditions

Plants were grown in Percival growth chambers (Percival, Boone, Iowa) under continuous illumination at 24 °C in Sunshine soil mix No. 1 (Sungro, Belleville, Washington). The light intensities used in this study are listed in Table 1. The *cch1* mutant was selected for its light-green phenotype from the Col-0 ecotype after EMS mutagenesis, and was backcrossed five times into the wild-type Col-0 background. Light intensity was measured with a spherical QSL-100 light meter which can measure diffused and scattered photosynthetic active radiation within the growth chamber (Biospherical Instruments, Inc., San Diego, California). Photoinhibitory light intensity was attained by placing plants outside in the middle of a sunny day, and the temperature was 24 ± 2 °C. For the moderate light to shaded light experiments, seedlings were grown for 7 days under low light, transferred to moderate light, and then moved into shaded light at either 1, 2, 4 days or 12 h prior to harvest when all plants were 17 days old. Zero-day controls remained at moderate light. For low to moderate light Chl-level experiments, seedlings were grown at low light, and transferred to moderate light 2, 4, 7, 14 days prior to harvest at 21 days. For low to moderate light RNA and protein experiments, seedlings were grown at low light, and transferred to moderate light 1, 2, 4, 7 days or 12 h prior to harvest at 14 days. In both cases, 0-day controls remained at low light. Leaf area measurements were made with a CI-202 Area Meter (CID, Inc., Camas, Washington).

Chl, Proto IX, and PChlide measurements

For Chl quantitation, approximately 10 mg of leaf tissue from well-irrigated plants was weighed, and then pigments were extracted into DMF for 3 h at room-temperature in the dark or overnight at 4 °C. Amounts of Chl *a* and Chl *b* (μg Chl/mg fresh weight) were determined spectrophotometrically using the equations of Porra et al. (1989), $n = 10$. For proto IX quantitation, 85 mg of leaf tissue was powdered in liquid N_2, and pigments were extracted into 1 ml 90% acetone:10% 0.1 M NH_4OH. After precipitation of plant material, hexane was used to remove Chl. F633 (proto IX) and F674 (Chl) were measured in both acetone and hexane extracts for each sample (excitation 406 nm) to normalize for the amount of F633 due to Chl, $n = 5$. For PChlide quantitation, 2–3 plants were extracted similarly. Acetone extracts were excited at 437 nm, and emission was detected at 630 nm. Hexane extracts were measured for absorbance at 668 nm, and used for normalization, $n = 4$.

Antibodies and immunoblot conditions

The CAO antibody was produced from a fragment of CAO that had been fused to the maltose binding protein using the pMAL fusion system (New England Biolabs, Beverly, Massachusetts). A partial *CAO* cDNA clone (103D24) was amplified with the following primers, 5′-GGAATTCAGTGTCCCAAGTTTGG TGAA-3′ and 5′-GCTCTAGATTAGCCGGAGAAAG GTAGTTT-3′, which amplify the region spanning S381 to G536, the C-terminal amino acid. The PCR product was ligated into the pMAL-c2 vector using XbaI and EcoRI, and the resulting plasmid insert was sequenced. The recombinant protein (4.3 mg) was purified according to manufacturer's instructions, cleaved with Factor Xa, and purified on an SDS-PAGE gel. Coomassie stained gel slices were sent to Antibodies, Inc. (Davis, California) for polyclonal antibody production in rabbits. The final antibody was used at a 1/2000 dilution. The antibody was found to give linear signal at 20, 30 and 40 μg of total leaf protein. In subsequent experiments, 30–40 μg of total leaf protein were loaded for western analysis. Total leaf proteins were extracted according to Barkan (1998). Plants of 4, 5, 14 or 17 days old were briefly ground in liquid N_2, and 75 μl of HbA buffer (0.28% β-mercaptoethanol, 10% sucrose, 100 mM Tris pH 7.4,

5 mM EDTA, 0.19% EGTA) was added and ground for 15–20 s. Then, 241.4 μl of HbA, 8.3 μl 60 mM PMSF and 0.3 μl of aprotinin were added, and grinding was continued. The protein extracts were stored at −80 °C, and protein concentrations were determined using the Bio-Rad dye binding assay (Bio-Rad, Inc., Hercules, California).

The Lhcb1, 2, 3, and 6 antibodies were obtained from L. Andrew Staehelin at the University of Colorado (Falbel and Staehelin 1992; Sigrist and Staehelin 1992, 1994). The Lhcb4 antibody was a gift of Xiao-Ping Li (University of California, Berkeley), and the Lhcb5 antibody was obtained from Agrisera, Inc., Vännäs, Sweden. Immunoblots conditions were described in Espineda et al. (1999). To test linearity, increasing amounts of wild-type thylakoids, prepared as described in Espineda et al. (1999), were immunoreacted with the five monospecific Lhcb antibodies. Immunoblots were scanned using an Epson Perfection 636U scanner, and the TIF images were then quantified using UN-SCAN-IT-GEL software (Silk Scientific, Orem, Utah). Time course experiments were repeated at least three times. Protein levels in thylakoid membrane preps were measured using the Bio-Rad dye-binding assay, and 20 μg of protein was loaded per sample.

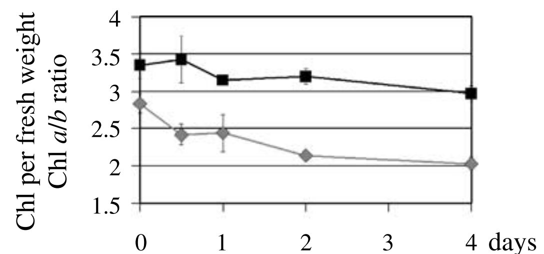
RNA gel blots

Total RNA was isolated from above ground rosette tissue as described in Brusslan and Tobin (1992) or using Trizol reagent (Invitrogen, Inc., Carlsbad, California). Electrophoresis, blotting, and methylene blue staining procedures were as described in Espineda et al. (1999). The *CAO* and *Lhcb1* probes were as described (Espineda et al. 1999). The *Elip1* and *Elip2* probes were made by PCR amplification of Col-0 genomic DNA. The *Elip1* primers were 5′-TGGATTAACCACTCGCAAGATCAAC AC-3′ and 5′-ATAACGTGCTCTCTTTTCCTTAGAC GAGT-3′ which amplified a 796 bp fragment. The *Elip2* primers were 5′-TCTCTCTTCTTCATCGATCT TCGGCTTTT-3′ and 5′-TCAATCTATGCGTTACAT AATCATTCTCCA-3′ which amplified a 870 bp fragment. All probes were gel-purified, and extracted using Geneclean (Bio 101, Inc., Vista, California). The final wash for all probes was at 65 °C in 0.1 × SSC/0.1% SDS. Bound probe was quantified using a Personal Molecular Imager FX (Bio-Rad, Inc., Hercules, California).

Table 1. Light intensities used in the experiments

Shaded light intensity	45 μmol photons m^{-2} s^{-1}
Low light intensity	95 μmol photons m^{-2} s^{-1}
Moderate light intensity	230 μmol photons m^{-2} s^{-1}
Photoinhibitory light intensity	1700 μmol photons m^{-2} s^{-1}

For shaded, low, and moderate light intensities, fluorescent bulbs were used. Photoinhibitory light intensity was achieved by placing plants outside on a sunny day. Light saturation curves (ΦII × irradiance) showed linearity between 52 and 180 μmol photons m^{-2} s^{-1}, and was saturated at an irradiance of 340 μmol photons m^{-2} s^{-1} in *Arabidopsis* (S. Rolfe, unpublished data). The lowest light intensity used in these measurements was 52 μmol photons m^{-2} s^{-1}, but it is likely that linearity is still maintained at 45 μmol photons m^{-2} s^{-1}.



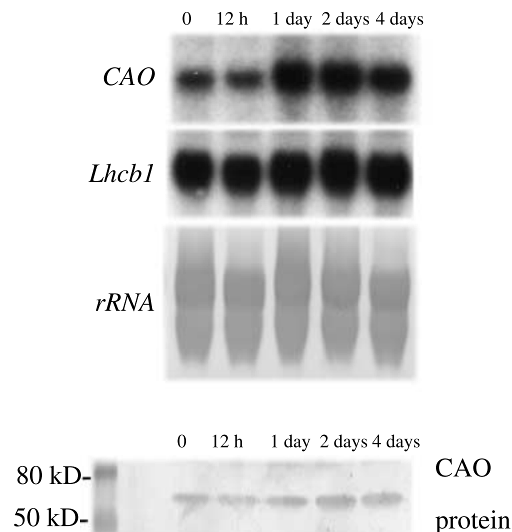


Figure 1. Chl, RNA and protein level in 17-day-old WT plants that have been transferred to shaded light intensity from moderate light intensity. In the top panel, the Chl *a*/*b* ratio is shown in black squares while the total Chl per fresh weight (μg Chl/mg fresh weight) is shown as gray diamonds. The middle panel shows RNA blots hybridized to *CAO* and *Lhcb* probes. Methylene blue stained ribosomal RNA was used to normalize the blots. A protein immunoblot, at the bottom, has been decorated with the CAO antibody. The staining on the immunoblot was quantified using UN-SCAN-IT software.

Figure 2. The left panel shows 17-day-old WT plants that had remained in moderate light while the right panel shows 17-day-old WT plants that had been transferred to shaded light intensity for 4 days. The scale is in cm. The plants were photographed together to avoid differences in shutter speed and aperture for an automatic digital camera.

Results

Transfer to shaded light intensity results in a decrease in the Chl a/b *ratio and an increase in* CAO *mRNA and protein levels*

Arabidopsis plants were grown at moderate light, and then transferred to shaded light conditions for 0, 12 h, 1 day, 2 days or 4 days, and all plants were harvested when they were 17 days old. These two light intensities were chosen because they are within the subsaturating portion of light saturation curves for *Arabidopsis* (see Table 1, legend). Plants grown at higher saturating light intensities were small and accumulated large amounts of anthocyanin indicating that they were undergoing significant stress, and thus were not suitable for experimentation. The top panel of Figure 1 shows that after 1 day, the Chl *a*/*b* ratio (black squares) displayed a small decrease which continued through day 4. At the same time, the μg total Chl/mg per fresh weight also decreased (gray diamonds). The decreased Chl *a*/*b* ratio was a result of a smaller loss in Chl *b* levels compared to Chl *a* levels under shaded light conditions which should lead to a relative increase in light-harvesting complexes. Figure 2 compares plants that remained in moderate light to those that were placed in shaded light for 4 days. The shaded light plants had smaller, lighter green leaves when compared to the darker green larger leaves that were observed in moderate light. Leaf area measurements indicated that the moderate light leaves were 22% larger (0.428 cm^2, SE = 0.11, $n = 10$) than the shaded leaves (0.351 cm^2, SE = 0.013, $n = 10$). In the bottom panels of Figure 1, *CAO* and *Lhcb1* mRNA and CAO protein levels are shown after transfer to shaded light intensity. *CAO* mRNA levels increased 2-fold and *Lhcb1* mRNA levels increased one- and one-half-fold after 1 day under shaded light conditions, and remained at an increased level for one more day before declining. CAO protein levels increased 2-fold after 2 days under shaded light intensity, and correlated to the decreased Chl *a*/*b* ratio.

Chl and Lhcb protein levels decreased when the cch1 *mutant was transferred to moderate light*

A decrease in Chl *b* levels was observed when the leaky Mg-chelatase mutant, *cch1*, was transferred from low to moderate light intensity. These light intensities were chosen because they result in little to no change in WT Chl levels, but significant changes in the Chl levels of the *cch1* mutant. These changes in the levels of Chl *a*, Chl *b* and the ratio between the two are shown in Figure 3A. In WT plants, both μg Chl *a*/mg fresh weight (black diamonds) and μg

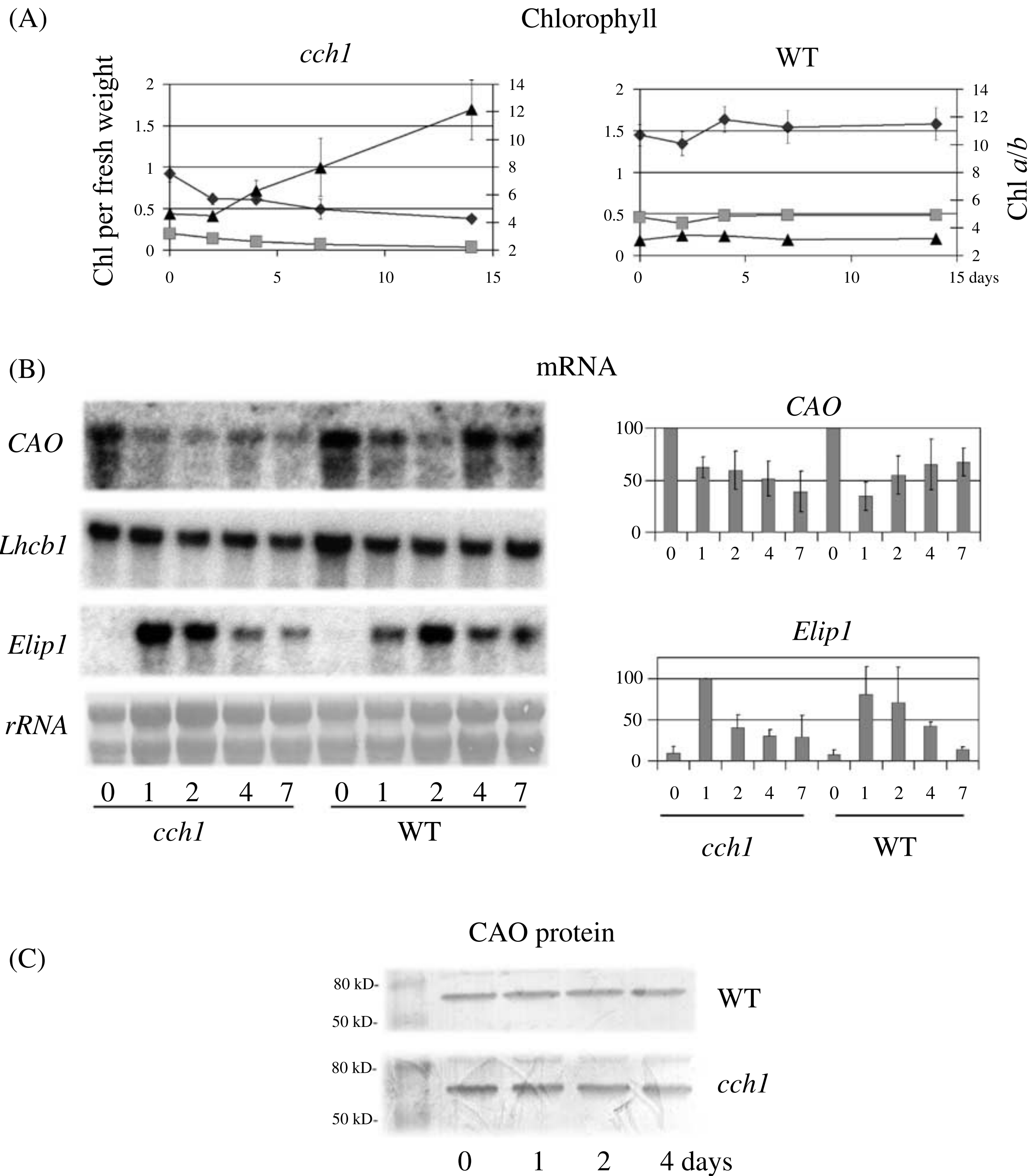


Figure 3. Chl, mRNA and protein levels in WT and *cch1* mutant plants that were transferred to moderate light intensity from low light intensity. (A) shows the Chl *a* levels (black diamonds) and Chl *b* levels (gray squares) and the Chl *a*/*b* ratio (black triangles) in both the *cch1* mutant and WT after transfer to moderate light intensity. In (B) RNA blots are shown on the left side, and quantitation of three experiments is shown on the right. (C) shows CAO protein immunoblots of WT and *cch1* after transfer to moderate light.

Chl *b*/mg fresh weight (gray squares) showed a slight decrease after 1 day at moderate light, but both Chl levels increased by 2 days, and then remained constant. The Chl *a*/*b* ratio (black triangles) showed an initial slight increase, but otherwise remained constant. In contrast, μg Chl *a*/mg fresh weight and μg Chl *b*/mg fresh weight both continuously declined in the *cch1* mutant, with Chl *b* levels decreasing

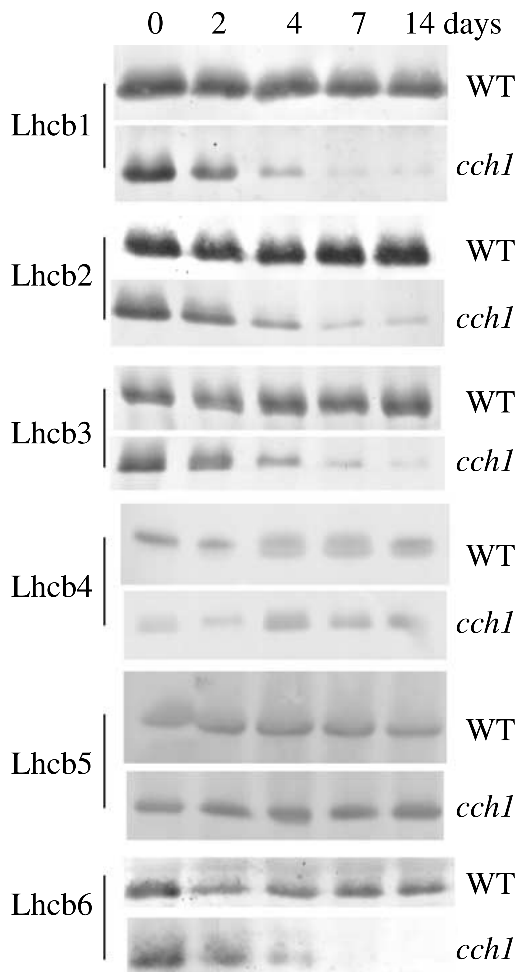


Figure 4. Protein immunoblot using Lhcb1-6 antibodies against equal amounts of protein from WT and *cch1* thylakoid membranes. In this series of experiments, plants were grown for 21 days, and were transferred to moderate light for the number of days indicated before harvest. Zero-day samples remained in low light for the duration of the experiment.

faster, and thus an increase in the Chl *a*/*b* ratio was observed.

The increase in the Chl *a*/*b* ratio was accompanied by the loss of Lhcb proteins from the thylakoid membrane. Lhcb proteins bind most of the Chl in the plant, and nearly all of the Chl *b*, and thus reduction in these proteins was expected. Immunoblot analysis in Figure 4 shows that Lhcb1, 2 and 3, which comprise the outer light-harvesting antennae were unstable when Chl levels were decreased. Lhcb6, part of the internal antennae was also unstable, while Lhcb4 and Lhcb5 levels increased despite the great loss of Chl. Stability of these two Lhcb proteins in the absence of Chl *b* has been observed previously (Peter and Thornber 1991; Król et al. 1995; Preiss and Thornber 1995). Quantitation of multiple immunoblots indicated that Lhcb1, 2, 3 and 6 all decayed with a half-time of 2 days. Immunoblots normalized on a per Chl basis, and reacted with the Lhcb2 antibody, showed a smaller reduction in immunostaining (data not shown), consistent with a 3-fold increase in the Chl *a*/*b* ratio seen in Figure 3A.

CAO *mRNA and protein levels both decrease after* cch1 *is transferred to moderate light*

WT and *cch1* plants were grown in low light, and transferred to moderate light intensity for 0, 1, 2, 4 and 7 days, and samples for mRNA and protein analysis were taken when all plants were 14 days old. In WT, *CAO* mRNA decreased after 1 day, but then recovered to near normal levels. A quantitation of *CAO* mRNA from three separate experiments is shown to the right of the RNA blot in Figure 3B. *Lhcb1* mRNA levels also decreased by 50% after transfer to moderate light intensity. *Elip* mRNA levels, encoding the early light inducible protein were also measured, as indicators of light stress. *Elip1* mRNA levels increased transiently, returning to low levels after seven days of moderate light treatment while *Elip2* mRNA levels were not induced by the mildly stressful moderate light treatment (data not shown). *Lhcb1* and *Elip1* mRNA levels followed a similar pattern in the *cch1* mutant, but the decrease in *CAO* mRNA was seen over the entire seven-day time course. Immunoblots of leaf extracts, shown in Figure 3C, indicated that CAO protein levels decreased in the *cch1* mutant by 50%, similar to the 60% decrease observed for *CAO* mRNA levels. These results indicate that CAO protein levels correlate to *CAO* mRNA levels, and suggest that the preference for Chl *a* production during limiting Chl synthesis could, in part, be regulated via reduced CAO protein levels.

Is the loss of Chl at moderate light intensity in the cch1 *mutant a result of reduced flux through the tetrapyrrole biosynthetic pathway?*

Two experiments were done to try to assess the flux of the tetrapyrrole biosynthetic pathway in the *cch1* mutant before and after transfer to moderate light. In the first experiment, shown in the top panel of Figure 5, protoporphyrin IX (PPIX) levels were measured in 20-day-old plants that remained in low light or were transferred to moderate light for 2 days. Pigments were extracted, and subjected to fluorescence analysis. PPIX levels were reduced to one-third WT

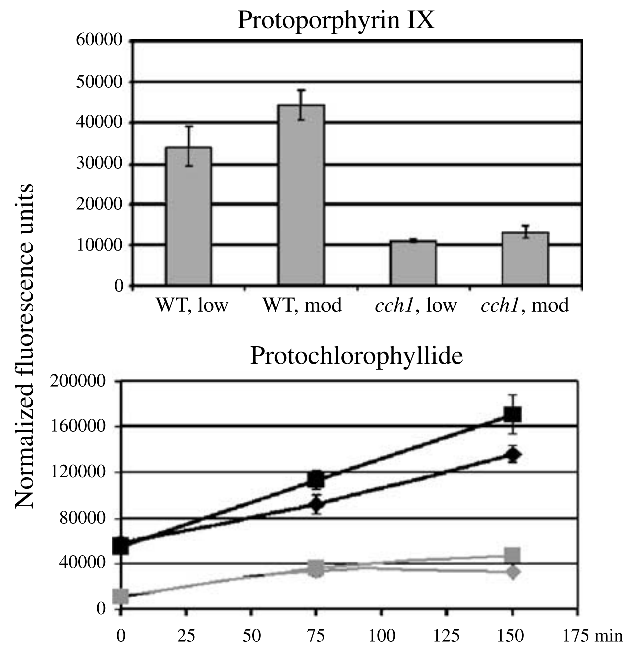


Figure 5. Protoporphyrin IX and PChlide levels were measured in both the WT and *cch1* mutants. In the top panel, PPIX levels were measured in plants that had been grown for 20 days in low light or transferred to moderate light for the last 2 days of growth. In the bottom panel, plants were grown similarly, but then transferred to darkness, and PChlide accumulation was measured at the indicated times. WT at moderate light is indicated by black squares, WT at low light by black diamonds, *cch1* at moderate light by gray squares and *cch1* at low light is indicated by gray diamonds.

levels in the *cch1* mutant, consistent with observations made in antisense *ChlH* tobacco plants (Papenbrock et al. 2000). Interestingly, PPIX levels were slightly increased in both WT and *cch1*, after transfer to moderate light.

The increase in PPIX under moderate light suggested that flux through the pathway was not further reduced when the *cch1* mutant was transferred to moderate light. To confirm this result, plants were treated in a similar manner, but after the 2 days at moderate light, the plants were transferred to darkness for 75 or 150 min. At these times pigments were extracted, and protochlorophyllide (PChlide) accumulation was measured. The results are shown in the bottom panel of Figure 5. In WT, PChlide increased linearly for 150 min, and the increase in moderate light had a slightly steeper slope than that for plants that remained in low light. Subsequent experiments indicated that the linear increase in PChlide continued for 225 min, and then tapered off by 300 min (data not shown). For *cch1*, the flux was substantially reduced, but again, a slight increase in PChlide accumulation was observed after two days of moderate light treatment. These experiments demonstrate that the loss of Chl in the *cch1* mutant after transfer to moderate light intensity was not a result of increased feedback inhibition of tetrapyrrole biosynthesis at higher light intensity (Cornah et al. 2003).

CAO protein levels decreased when plants were exposed to very high light intensity

To determine whether CAO protein levels fluctuated when *Arabidopsis* plants were subject to photoinhibitory conditions, plants were grown in low light for 17 days before transfer to very high light intensity for 3 h. During this treatment, samples were taken every 30 min for RNA and at the end of the time course for protein and chlorophyll. In Figure 6, *Elip1* and *Elip2* mRNA levels, increased rapidly after transfer to very high light intensity with *Elip1* mRNA becoming detectable after 30 min and accumulating to greater levels than *Elip2*. Rapid induction of these genes has been correlated with exposure to high light intensity in *Arabidopsis* and other species (Adamska 1997), and it has been proposed that the Elip proteins sequester unbound Chl during photoinhibition (Hutin et al. 2003). The *Lhcb1* mRNA levels were decreased by 50%, and this decrease was complete after 2.5 h. This is similar to the decrease observed in barley (Montané et al.

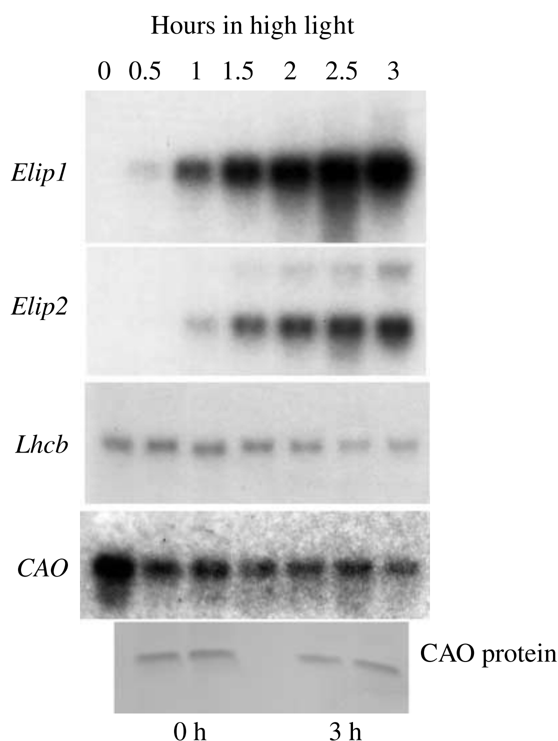


Figure 6. mRNA and protein levels during photoinhibitory conditions. Seventeen-day-old plants were grown in low light and transferred to photoinhibitory light intensity for the number of hours shown. RNA gel blots were hybridized to the *Elip1*, *Elip2*, *Lhcb1* and *CAO* probes. The bottom panel shows protein immunoblots using the CAO antibody.

1998) and *Chlamydomonas* (Teramoto et al. 2002). *CAO* mRNA levels also decreased, with kinetics similar to *Lhcb1* mRNA. Immunoblot analysis indicated that CAO protein levels decreased by 50% after 3 h of very high light treatment. Thus after transfer to very high light, a decrease in *CAO* mRNA was again reflected in CAO protein levels. After 3 h of photoinhibition, the total Chl per fresh weight decreased by 18% (2.55 ± 0.08–$2.08 \pm 0.05\,\mu g\,mg^{-1}$ fresh weight) while the Chl *a*/*b* ratio decreased by 6% (3.03 ± 0.09–2.84 ± 0.14). The significant decrease in total Chl during this short period may have been regulated, in part, by a decrease in *CAO* mRNA levels.

Discussion

The relationship between *CAO* mRNA, CAO protein, and Chl levels was explored in this work. In two different types of experiments, changes in the Chl *a*/*b* ratio correlated to changes in *CAO* mRNA and CAO protein levels. The changes in *CAO* mRNA and protein levels were not rapid, but did coincide with the changes in Chl *a*/*b* ratios. This suggests that changes in *CAO* mRNA and protein levels can affect Chl *b* levels, and are part of a plant's long-term acclimation response to changes in light intensity and Chl availability.

When plants were moved to shaded light intensity, the Chl *a*/*b* ratio decreased after 1 day and *CAO* mRNA levels increased at the same time, but CAO protein levels did not increase until one day later. These results suggest that the initial relative increase in Chl *b* levels was not the result of greater amounts of CAO protein. However, under longer term acclimation to lower light intensity, higher CAO protein levels might be important for maintaining increased levels of Chl *b*. Interestingly, the levels of total Chl/fresh weight tissue also decreased when plants were moved to shaded light intensity. A decrease in both total Chl/mg fresh weight and the Chl *a*/*b* ratio has been observed for certain species when moderate light and shaded light intensity grown plants have been compared (Murchie and Horton 1997).

We searched for other conditions that would perturb the Chl *a*/*b* ratio in *Arabidopsis*. In pea, transfer to far-red enriched light favors an increase in LHCII, resulting in a greater amount of Chl *b* (Kim et al. 1993). Unfortunately, *Arabidopsis* plants grown under far-red light under two different light intensities did not display any changes in their Chl *a*/*b* ratio (Walters and Horton 1994).

The *cch1* mutant is a leaky Chl biosynthesis mutant which has a more pronounced chlorina (yellow-green) phenotype under moderate light intensity. It is possible that the reaction center apoproteins have greater affinity for Chl *a* than do Lhcb apoproteins, and thus, under conditions of limiting Chl synthesis, reaction center synthesis is favored, leaving little excess Chlide *a* for conversion into Chlide *b*. In the *cch1* mutant, transfer to moderate light intensity resulted in a large increase in the Chl *a*/*b* ratio and decreased *CAO* mRNA and protein levels. This suggests that the synthesis of Chl *b* is not solely regulated by Chlide *a* availability, but that adjustment to Chl availability can take place at the level of CAO protein abundance.

The initial drop in *CAO* mRNA levels was observed in both WT and *cch1*, but *CAO* mRNA levels remained low only in the *cch1* mutant. The mutant also exhibited reduced CAO protein levels, which became apparent after one day at moderate light intensity. The initial drop in *CAO* mRNA levels in WT was not accompanied by a decrease in protein levels indicating that mRNA and protein levels do not always correlate.

After 3 h at photoinhibitory light intensity, *CAO* mRNA and CAO protein levels both decreased, and a substantial (18%) decrease in Chl/fresh weight was observed. Under photoinhibitory conditions, the D1 protein is damaged, and cannot be replaced at an adequate pace (Barber and Andersson 1992; Aro et al. 1993). Both *Elip* genes were highly expressed under these conditions, suggesting that the plants were responding to light stress under the photoinhibitory light conditions. During light stress, the production of $^1O_2$ is especially damaging to the highly unsaturated lipids of the thylakoid membrane (Niyogi 1999), and the thylakoid-bound LHCs become dismantled. The Chl bound to the LHCs is likely scavenged by Elip proteins as well as subjected to photodynamic turnover. A reduction in the size of the LHC could provide photoprotection by reducing the amount of absorbed photons. However, recent data in *Chlamydomonas reinhardtii* suggest that photooxidative damage in a zeaxanthin/lutein double mutant is only slightly less when accumulation of the LHCII is severely reduced by the *cbn1* mutation (Baroli et al. 2003). Thus the functional significance of the 18% drop in total Chl levels after 3 h at photoinhibitory light intensity still needs to be established.

The cause of the light-intensity dependent conditional chlorina phenotype was also explored. The reduction in Chl resulted from loss of the peripheral Lhcb proteins, Lhcb1, 2 and 3, which bind the majority of Chl (Thornber et al. 1994) as well as loss of one of the more internal proteins, Lhcb6. Lhcb4 and Lhcb5 appeared to be stable. Similar stability of the Lhcb proteins has been observed in other vascular plants with defects in Chl *b* synthesis (Peter and Thornber 1991; Król et al. 1995; Preiss and Thornber 1995). It is possible that a transient accumulation of PPIX in the *cch1* Mg-chelatase mutant could be phototoxic at a higher light intensity, and this might result in increased feedback inhibition of aminolevulinate biosynthesis. This was not observed in two separate experiments that measured PPIX levels and PChlide accumulation after transfer to the dark. In both cases, a small increase in the flux through the tetrapyrrole biosynthetic pathway was apparent at the higher light intensity. An increase in feedback inhibition has thus been ruled out as a cause of the higher light intensity conditional chlorina phenotype. An increased demand for Chl at higher light intensity, due to increased growth and/or perhaps faster turnover of the Lhcb proteins, is the likely cause of the conditional phenotype.

Previously, *CAO* overexpression was shown to result in increased Chl *b* synthesis during the early phases of greening, suggesting that during this developmental transition, *CAO* mRNA levels were limiting. In mature plants, however, *CAO* overexpression only had a small effect on Chl *b* levels (Tanaka et al. 2001). Our data indicate that endogenous *CAO* mRNA and protein levels change in relation to the Chl *a*/*b* ratio, suggesting that the size of the light-harvesting complex is regulated, in part, by changes in *CAO* mRNA levels. Other levels of regulation could occur via interaction between Lhcb apoproteins and the CAO protein, as suggested by enhanced Chl *b* synthesis in cyanobacteria that coexpress both CAO and Lhcb (Xu et al. 2001).

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References

Adamska I (1997) ELIPs-light-induced stress proteins. Physiol Plant 100: 794–805

Andersson J, Wentworth M, Walters RG, Howard CA, Ruban AV, Horton P and Jansson S (2003) Absence of the Lhcb1 and Lhcb2 proteins of the light-harvesting complex of Photosystem II-effects on photosynthesis, grana stacking and fitness. Plant J 35: 350–361

Aro E-M, Virgin I and Andersson B (1993) Photoinhibition of Photosystem II: inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134

Barber J and Andersson B (1992) Too much of a good thing: light can be bad for photosynthesis. Trends Biochem 17: 61–66

Barkan A (1998) Approaches to investigating nuclear genes that function in chloroplast biogenesis in land plants. Meth Enzymol 297: 38–57

Baroli I, Do AD, Yamane T and Nigyogi K (2003) Zeaxanthin accumulation in the absence of a functional xanthophylls cycle protects *Chlamydomonas reinhardtii* from photooxidative stress. Plant Cell 15: 992–1008

Brusslan JA and Tobin EM (1992) Light-independent developmental regulation of *cab* gene expression in *Arabidopsis thaliana* seedlings. Proc Natl Acad Sci USA 89: 7791–7795

Cornah JE, Terry MJ and Smith AG (2003) Green or red: what stops the traffic in the tetrapyrrole pathway? Trends Plant Sci 8: 224–230

Droppa M, Ghirardi ML, Horvath G and Melis A (1988) Chlorophyll *b* deficiency in soybean mutants. II. Thylakoid membrane

development and differentiation. Biochem Biophys Acta 932: 138–145

Espineda CE, Linford AS, Devine D and Brusslan JA (1999) The *AtCAO* gene, encoding chlorophyll *a* oxygenase, is required for chlorophyll *b* synthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96: 10507–10511

Falbel TG and Staehelin LA (1992) Species-related differences in the electrophoretic behavior of CP 29 and CP 26: an immunochemical analysis. Photosynth Res 34: 249–262

Falbel TG and Staehelin LA (1996) Partial blocks in the early steps of the chlorophyll synthesis pathway: a common feature of chlorophyll *b*-deficient mutants. Physiol Plant 97: 311–320

Flachmann R and Kühlbrandt W (1995) Accumulation of plant antenna complexes is regulated by post-transcriptional mechanisms in tobacco. Plant Cell 7: 149–160

Greene BA, Allred DR, Morishige DT and Staehelin LA (1988) Hierarchical response of light-harvesting chlorophyll-proteins in a light-sensitive chlorophyll *b*-deficient mutant of maize. Plant Physiol 87: 357–364

Hutin C, Nussaume L, Moise N, Moya I, Kloppstech K and Havaux M (2003) Early light-induced proteins protect *Arabidopsis* from photooxidative stress. Proc Natl Acad Sci USA 100: 4921–4926

Kim JH, Glick RE and Melis A (1993) Dynamics of photosystem stoichiometry adjustment by light quality in chloroplasts. Plant Physiol 102: 181–190

Knoetzel J and Simpson D (1991) Expression and organization of antenna proteins in the light- and temperature-sensitive barley mutant chlorina^{104}. Planta 185: 111–123

Król M, Spangfort MD, Huner NPA, Öquist G, Gustafsson P and Jansson S (1995) Chlorophyll *a*/*b*-binding proteins, pigment conversions, and early light-induced proteins in a chlorophyll *b*-less barley mutant. Plant Physiol 107: 873–883

Larsson UK, Anderson JM and Andersson B (1987) Variations in the relative content of the peripheral and inner light-harvesting chlorophyll *a*/*b*-protein complex (LHCII) subpopulations during thylakoid light adaptation and development. Biochem Biophys Acta 894: 69–75

Leong T-Y and Anderson JM (1984) Adaptation of the thylakoid membranes of the pea chloroplasts to light intensities. I. Study on the distribution of chlorophyll–protein complexes. Photosynth Res 5: 105–115

Masuda T, Polle JEW and Melis A (2002) Biosynthesis and distribution of chlorophyll among the photosystems during recovery of the green alga *Dunaliella salina* from irradiance stress. Plant Physiol 128: 603–614

Masuda T, Tanaka A and Melis A (2003) Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll *a* oxygenase (*CAO*) and *Lhcb* gene expression. Plant Mol Biol 51: 757–771

Mochizuki N, Brusslan JA, Larkin R, Nagatani A and Chory J (2001) *Arabidopsis* genome uncoupled 5 (*gun5*) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc Natl Acad Sci USA 98: 2053–2058

Montané M-H, Tardy F, Kloppstech K and Havaux M (1998) Differential control of xanthophylls and light-induced stress proteins, as opposed to light-harvesting chlorophyll *a*/*b* proteins, during photosynthetic acclimation of barley leaves to light irradiance. Plant Physiol 118: 227–235

Murchie EH and Horton P (1997) Acclimation of photosynthesis to irradiance and spectral quality in British plant species: chlorophyll content, photosynthetic capacity and habitat preference. Plant Cell Environ 20: 438–448

Niyogi KK (1999) Photoprotection revisited: genetic and molecular approaches. Ann Rev Plant Physiol Plant Mol Biol 50: 333–359

Oster U, Bauer CE and Rüdiger W (1997) Characterization of chlorophyll *a* and bacteriochlorophyll *a* synthases by heterologous expression in *Escherichia coli*. J Biol Chem 272: 9671–9676

Oster U, Tanaka R, Tanaka A and Rüdiger W (2000) Cloning and functional expression of the gene encoding the key enzyme for chlorophyll *b* biosynthesis (CAO) from *Arabidopsis thaliana*. Plant J 21: 305–310

Papenbrock J, Mock HP, Tanaka R, Kruse E and Grimm B (2000) Role of magnesium chelatase activity in the early steps of the tetrapyrrole biosynthetic pathway. Plant Physiol 122: 1161–1169

Peter GF and Thornber JP (1991) Biochemical composition and organization of higher plant Photosystem II light-harvesting pigment-proteins. J Biol Chem 266: 16745–16754

Porra RJ, Thompson WA and Kriedmann PA (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochem Biophys Acta 975: 384–394

Preiss S and Thornber JP (1995) Stability of apoproteins of light-harvesting complex I and II during biogenesis of thylakoids in the chlorophyll *b*-less barley mutant chlorina f2. Plant Physiol 107: 709–717

Rüdiger W (2002) Biosynthesis of chlorophyll *b* and the chlorophyll cycle. Photosynth Res 74: 187–193

Satoh S, Ikeuchi M, Mimuro M and Tanaka A (2001) Chlorophyll *b* expressed in cyanobacteria functions as a light-harvesting antenna in Photosystem I through flexibility of the proteins. J Biol Chem 276: 4293–4297

Sigrist M and Staehelin LA (1992) Identification of type 1 and type 2 light-harvesting chlorophyll a/b-binding proteins using monospecific antibodies. Biochem Biophys Acta 1098: 191–200

Sigrist M and Staehelin LA (1994) Appearance of Type 1, 2, and 3 light-harvesting complex II and light-harvesting complex I proteins during light-induced greening of barley (*Hordeum vulgare*) etioplasts. Plant Physiol 104: 135–145

Sukenik A, Bennett J, Mortain-Bertrand A and Falkowski PG (1990) Adaptation of the photosynthetic apparatus to irradiance in *Dunaliella tertiolecta*. Plant Physiol 92: 891–898

Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshida K and Okada K (1998) Chlorophyll *a* oxygenase (*CAO*) is involved in chlorophyll *b* formation from chlorophyll *a*. Proc Natl Acad Sci USA 95: 12719–12723

Tanaka R, Koshino Y, Sawa S, Ishigura S, Okada K and Tanaka A (2001) Overexpression of chlorophyllide *a* oxygenase (CAO) enlarges the antenna size of Photosystem II in *Arabidopsis thaliana*. Plant J 26: 365–373

Teramoto H, Nakamori A, Minagawa J and Ono T-A (2002) Light-intensity-dependent expression of the *Lhc* gene family encoding light-harvesting chlorophyll-*a*/*b* proteins of Photosystem II in *Chlamydomonas reinhardtii*. Plant Physiol 130: 325–333

Thornber JP, Cogdell RJ, Chitnis P, Morishige DT, Peter GF, Gómez SM, Anandan S, Preiss S, Dreyfuss BW, Lee A, Takeuchi T and Kerfield C (1994) Antenna pigment–protein complexes of higher plants and purple bacteria. Adv Mol Cell Biol 10: 55–118

Tomitani A, Okada K, Miyashita H, Matthijs HCP, Ohno T and Tanaka A (1999) Chlorophyll *b* and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature 400: 159–162

Walters RG and Horton P (1994) Acclimation of *Arabidopsis thaliana* to the light environment: changes in composition of the photosynthetic apparatus. Planta 195: 248–256

Xu H, Vavilin D and Vermaas W (2001) Chlorophyll *b* can serve as the major pigment in functional Photosystem II complexes of cyanobacterium. Proc Natl Acad Sci USA 98: 14168–14173